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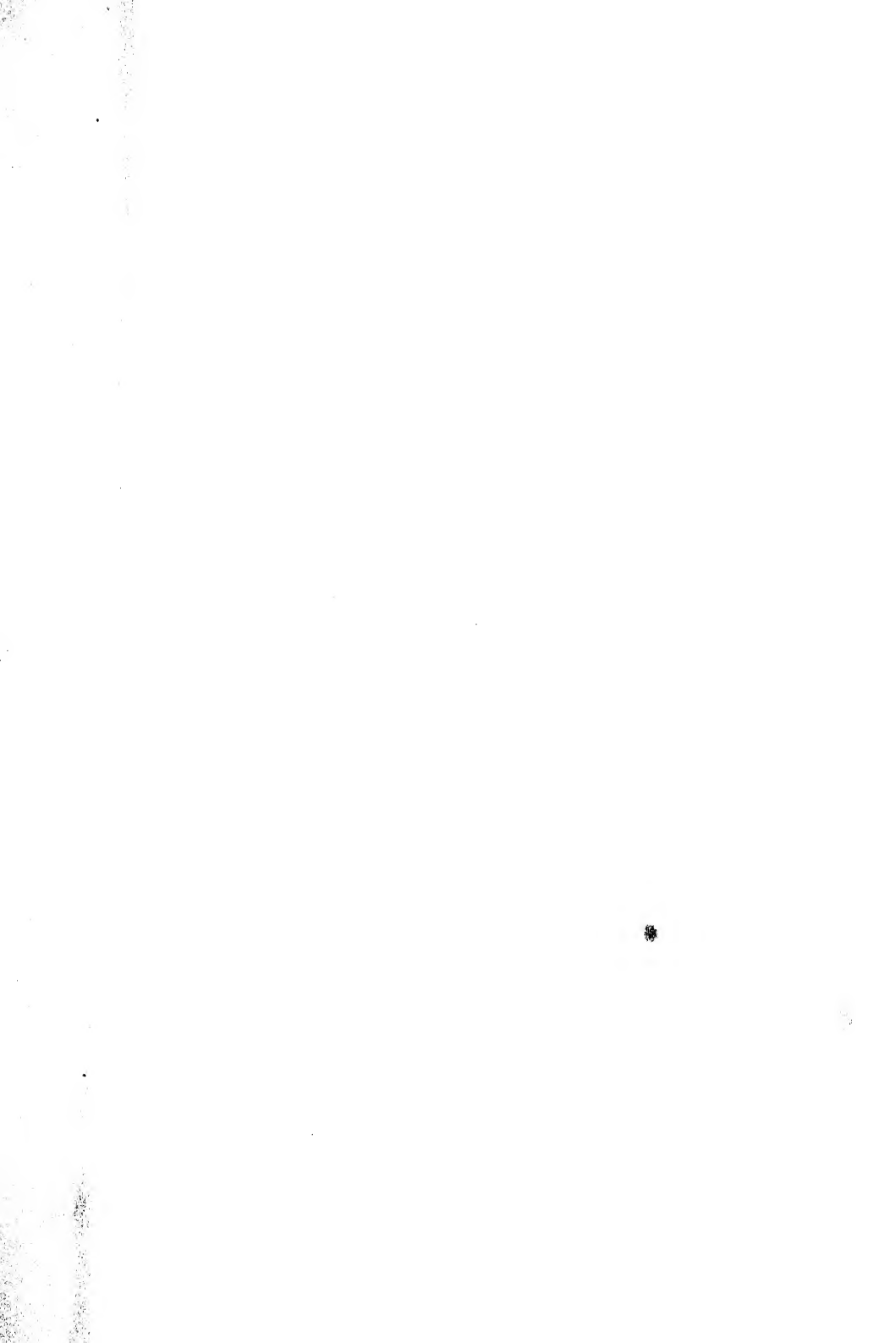
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No. 1

THE EFFECT OF pH ON HUMORAL STIMULATION OF STRIATED MUSCLE AND ITS APPLICATION TO THE CHEMICAL CONTROL OF BREATHING¹

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The rectus abdominis muscle of the frog immersed in an acetylcholine solution partially saturated with carbon dioxide contracts more vigorously than when immersed in a non-carbonated acetylcholine solution of equal strength (Gesell, Mason and Brassfield, 1944). These findings are in agreement with the acid-neurohumoral theory of nervous integration which holds that acid retards the hydrolysis of acetylcholine normally liberated at active cholinergic nerve terminals thus increasing the intensity of humoral stimulation (Gesell, Brassfield and Hamilton, 1942).

The simplicity of structure of striated muscle and its neuromyal junctions offers exceptional opportunities for studying the principles of neurohumoral mediation. Acetylcholine of an ambient solution enters the muscle by the process of diffusion. According to the results on the rectus abdominis it is believed that acetylcholine is enabled to penetrate the muscle more deeply by virtue of the protective action of carbon dioxide. Greater numbers of muscle fibers are stimulated and the strength of contraction is increased. Experiments were accordingly conducted on other skeletal muscles of amphibians and reptiles to determine the manner in which the response of muscle to humoral mediation is modified by hydrogen ion concentration. To our surprise it was found that the effects of carbon dioxide upon humoral mediation of contraction varied with the type of muscle tested. Contractions of muscles which are primarily respiratory were potentiated while those of locomotor muscles were diminished by carbon dioxide (Finerty and Gesell, 1944, 1945). This unexpected differential action is studied in greater detail in the present experiments on skeletal muscles of the frog, turtle and alligator.

METHODS. The muscles were excised, mounted in an especially devised container and attached to a recording muscle lever. The container was equipped with a three-way stopcock for quickly flooding and changing the solutions about the muscle. Three types of Ringer-Locke solutions were used: 1, standard

¹ These experiments were supported by a research grant from the Rackham Foundation.

Ringer-Locke solution; 2, bicarbonate-free Ringer-Locke solution, and 3, Ringer-Locke solution plus excess sodium bicarbonate. The muscle was flooded for one minute in an acetylcholine containing solution (1, 2 or 3). It was then quickly drained and reflooded with an acetylcholine-free solution. Ten minutes following this control observation the muscle was reflooded for one minute with the test solution. This solution, containing the same amount of acetylcholine as the control solution, was modified by adding the desired amount of acid (carbon dioxide, lactic, phosphoric, or hydrochloric acid) or of base (sodium bicarbonate). The muscle was then drained as before and reflooded with acetylcholine-free solution after which the initial control observation was repeated. (See figs. 1A, B and C, and 1F, G and H.) The hydrogen ion concentration of each solution was determined with the glass electrode.

Although the pH of the solutions in which the muscles were suspended was carefully controlled, it must be remembered that changes in the pH of the environment do not necessarily produce corresponding changes in the pH of the interior of the muscle fibers or motor end plates. Differential permeability of the muscle and end plate membranes to CO_2 and NaHCO_3 as well as the intracellular buffering action are factors modifying the influence of the environmental solutions. Since carbon dioxide penetrates tissues freely (Jacobs, 1920) it will tend to turn muscle more acid, but the degree of change of intracellular pH which occurs on immersion of the muscle in a carbon dioxide-containing solution will be determined by the relative rates of penetration of CO_2 and NaHCO_3 and the buffering power of the tissue itself.

It is well to remember that the contraction of a muscle stimulated by acetylcholine is the summed effect of rhythmic twitches of those individual muscle fibers reached by a threshold concentration of the neurohumor. The contraction is, therefore, a composite response in which the number of muscle fibers participating depends upon the depth of penetration of the activating humor. Throughout this discussion, height of contraction refers to the excursion of the recording lever, and duration of contraction to the period during which the muscle continues to shorten. The complete response of the muscle, however, consists of two phases: 1, a period of increasing height of contraction, during which the neurohumor is penetrating the muscle and activating an increasing number of fibers; and 2, a period of decreasing height of contraction, or relaxation, when the number of fibers active is reduced.

RESULTS. *Effect of carbon dioxide on contraction of respiratory muscles.* Characteristic results obtained on the rectus abdominis muscle of the frog in an acetylcholine solution partially saturated with carbon dioxide are shown in figures 1F, G and H. Contractions 1F and 1H are in response to immersion in a bicarbonate-free solution of acetylcholine (1-100,000). They show typically smooth contraction curves in which the period of contraction persists for a short time after the muscle is reflooded with acetylcholine-free solution. Contractions are eventually terminated by a slow relaxation. The addition of 50 volumes per cent of carbon dioxide to the acetylcholine solution in figure 1G prolongs the period of contraction, increases its height and prolongs the relaxation phase.

Progressively increasing saturation of the muscle with carbon dioxide produces progressively increasing potentiation of contraction. This is demonstrated in figures 2B, 2C, 2D and 2E in which 10, 30, 50 and 70 volumes per



Fig. 1. Response of the rectus abdominis muscle of the frog to acetylcholine (1-100,000) in Ringer Locke solution modified by NaHCO_3 and CO_2 . Weakening of response produced by addition of 0.15 per cent NaHCO_3 to a bicarbonate-free Ringer-Locke solution is shown in B. A and C are control contractions in response to acetylcholine in bicarbonate-free solution. Potentiation of contraction produced by the addition of 0.15 per cent NaHCO_3 and 50 volumes per cent CO_2 to a bicarbonate-free solution is shown in E, with D as a control contraction in non-carbonated acetylcholine solution. Potentiation of contraction produced by addition of 50 volumes per cent CO_2 to a bicarbonate-free solution is shown in G. F and H are control contractions in response to acetylcholine in bicarbonate-free solution. Equally strong potentiation occurring in 1E and 1G at widely differing pH values indicates the importance of the pCO_2 of the ambient solution.

Fig. 2. Progressive potentiation of contraction of the rectus abdominis muscle of the frog produced by progressively increased CO_2 tension. Each trial consists of flooding the muscle for one minute with acetylcholine (1-1,000,000) in bicarbonate-free Ringer-Locke solution to which 10, 30, 50 and 70 volumes per cent CO_2 were added in B, C, D and E, respectively. A and F are control contractions in non-carbonated bicarbonate-free acetylcholine solutions.

cent of carbon dioxide were added, respectively, to a bicarbonate-free solution of acetylcholine (1-1,000,000). The height of contractions mounts with the first three additions of carbon dioxide, a maximum increase occurring at 50 volumes per cent of carbon dioxide. At this optimal carbon dioxide tension

there was a marked lengthening of the period of relaxation as well as that of contraction. Prolongation of contraction may be regarded as an after-effect of the acetylcholine and carbon dioxide retained by the muscle at the moment of reflooding with CO_2 free solution. This effect became more apparent on changing to a 70 per cent carbon dioxide solution. Since the muscle was lightly weighted, the distance it was artificially returned to the base-line gives indication of the extent of the after-contraction.

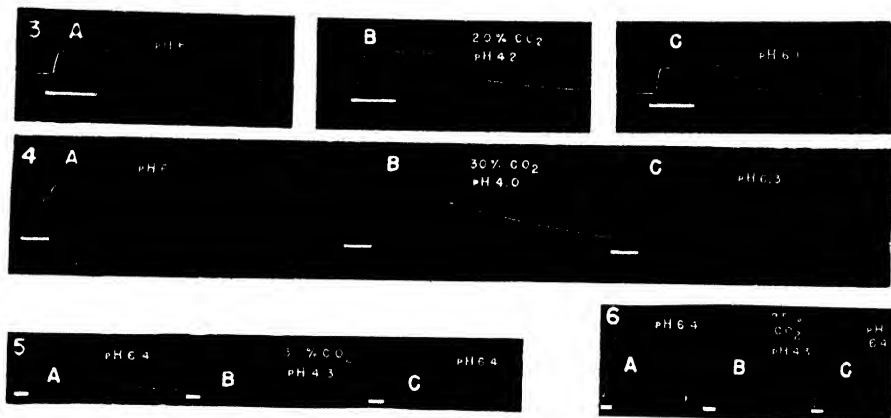


Fig. 3. Potentiation of contraction of the mylohyoid muscle of the frog produced by addition of 20 volumes per cent CO_2 to a bicarbonate-free Ringer-Locke solution containing acetylcholine (1-25,000) is shown in B. A and C are control contractions in non-carbonated acetylcholine solutions.

Fig. 4. Potentiation of contraction of the mylohyoid muscle of the turtle produced by addition of 30 volumes per cent CO_2 to a bicarbonate-free Ringer-Locke solution containing acetylcholine (1-400,000) is shown in B. A and C are control contractions in non-carbonated acetylcholine solutions.

Fig. 5. Potentiation of contraction of the genioid muscle of the bullfrog produced by addition of 30 volumes per cent CO_2 to a bicarbonate-free Ringer-Locke solution containing acetylcholine (1-500,000) is shown in B. A and C are control contractions in non-carbonated acetylcholine solutions.

Fig. 6. Potentiation of contraction of a strip of muscle from the diaphragm of an alligator produced by addition of 25 volumes per cent CO_2 to a bicarbonate-free Ringer-Locke solution containing acetylcholine (1-100,000) is shown in B. A and C are control contractions in non-carbonated acetylcholine solutions.

The response of the mylohyoid muscle of a frog to acetylcholine was potentiated by carbon dioxide in a manner similar to that of the rectus abdominis. Figure 3B shows the effect of adding 25 volumes per cent carbon dioxide to a bicarbonate-free acetylcholine solution (1-25,000). Compare with the contractions 3A and 3C occurring in carbon dioxide free solution.

Similar but somewhat smaller potentiation was observed on the mylohyoid muscle of the turtle (see fig. 4). The increases in height and duration of contraction were produced by the addition of 50 volumes per cent of carbon dioxide to a bicarbonate-free solution of acetylcholine (1-400,000).

Potential of contraction of the geniohyoid muscle of the bull frog illustrated in figure 5B was produced by the addition of 30 volumes per cent of carbon dioxide to a bicarbonate-free solution of acetylcholine (1-500,000).

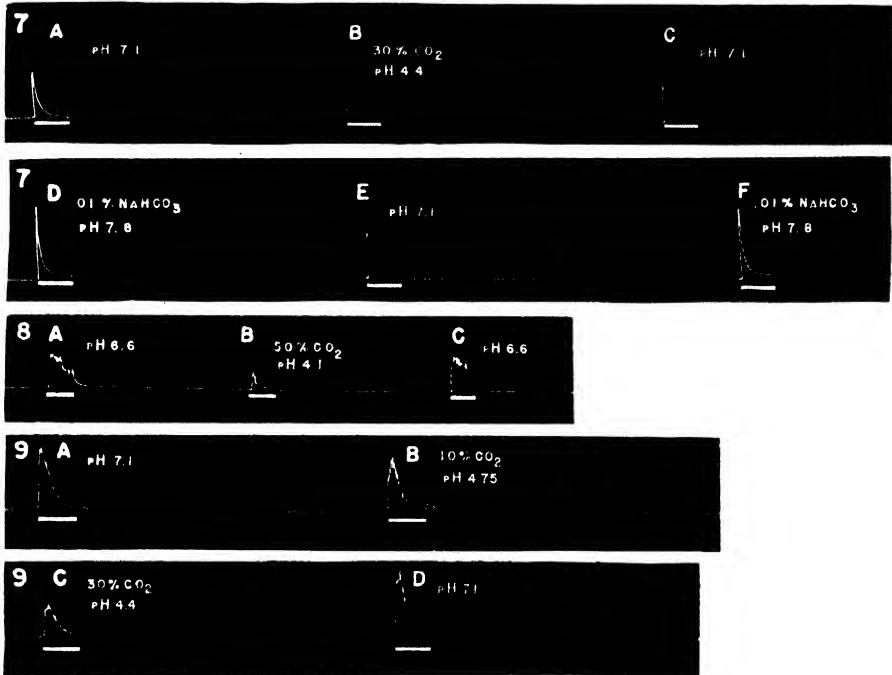


Fig. 7. Response of the sartorius muscle of the frog to acetylcholine (1-50,000) in Ringer-Locke solution modified by NaHCO_3 and CO_2 . Weakening of response in B produced by addition of 30 volumes per cent CO_2 to a bicarbonate-free Ringer-Locke solution. Potentiation of contraction by addition of 0.01 per cent NaHCO_3 to bicarbonate-free solutions is shown in D and F. A, C and E are control contractions in response to acetylcholine in a bicarbonate-free solution.

Fig. 8. Weakening of contraction of the peroneus muscle of the frog produced by addition of 50 volumes per cent CO_2 to a bicarbonate-free Ringer-Locke solution containing acetylcholine (1-30,000) is shown in B. A and C are control contractions in non-carbonated acetylcholine solutions.

Fig. 9. Weakening of response of the gracilis muscle of the frog to acetylcholine (1-50,000) produced by addition of 10 and 30 volumes per cent CO_2 to bicarbonate-free Ringer-Locke solutions in B and C, respectively. A and D are control contractions in non-carbonated acetylcholine solutions.

Potentiation of contraction of a strip of muscle excised from the diaphragm of the alligator, illustrated in figure 6, is the effect of 25 volumes per cent of CO_2 added to a bicarbonate-free solution of 1-100,000 acetylcholine.

Effect of carbon dioxide on contraction of locomotor muscles. As previously reported, the addition of carbon dioxide to an acetylcholine solution resulted in a diminution of contraction rather than in a potentiation. Another striking

difference between the respiratory and locomotor muscles, as exemplified by the rectus abdominis and sartorius muscles, is seen in the shape and duration of the contraction curve. When the sartorius muscle is flooded with acetylcholine solution it contracts sharply. There is usually a spike-like contraction which is followed by a rapid relaxation while the muscle is still exposed to the acetylcholine solution (see fig. 7). This stands in marked contrast to the smooth and prolonged curve of contraction of the rectus abdominis which reaches its greatest height during the initial stages of reflooding with acetylcholine-free

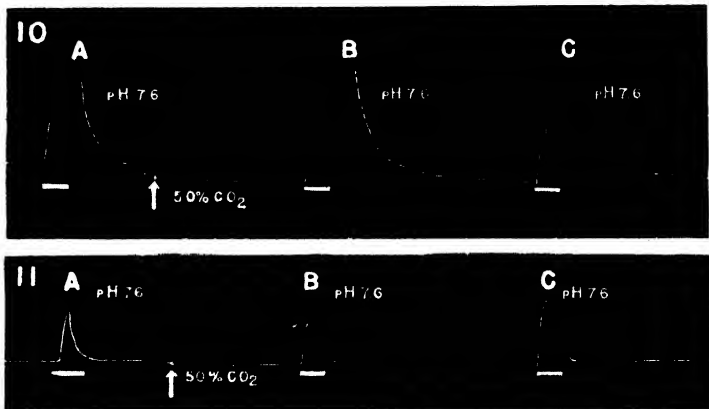


Fig. 10. Potentiation of response of the rectus abdominis muscle of the frog to acetylcholine by pre-acidulation with CO_2 . The contractions in A, B and C are all in response to flooding of the muscle for one minute in Ringer-Locke solution containing 1-200,000 acetylcholine. Immediately preceding B the muscle was flooded for 5 minutes with Ringer-Locke solution containing 50 volumes per cent CO_2 .

Fig. 11. Weakening of response of the sartorius muscle of the frog to acetylcholine by pre-acidulation with CO_2 . The contractions in A, B and C are all in response to flooding of the muscle for one minute in Ringer-Locke solution containing 1-100,000 acetylcholine. Immediately preceding B the muscle was flooded for 5 minutes with Ringer-Locke solution containing 50 volumes per cent CO_2 .

solution. Quick contraction and relaxation is, in general, the typical response of locomotor muscles to acetylcholine, while slower, more prolonged and stronger contractions characterize the rectus abdominis and other respiratory muscles.

The weakening effect produced by 30 volumes per cent of carbon dioxide added to a bicarbonate-free acetylcholine solution is illustrated in the contraction of the sartorius muscle in figure 7B. Compare 7B with 7A and 7C. Addition of sodium bicarbonate in place of carbon dioxide resulted in higher contractions. Compare 7D and 7F with 7E.

Contractions of the peroneus muscle were affected in the same way as those of the sartorius muscle. Carbon dioxide diminished the response to acetylcholine whether the addition was made to a bicarbonate-free or standard Ringer-Locke solution (fig. 8).

The response of the gracilis muscle of the frog to increased carbon dioxide concentration of the environment varied with the ambient solution used. When carbon dioxide was added to a buffered solution there was a slight increase in height of contraction but when added to an unbuffered solution, the height of contraction was diminished (fig. 9).

Preacidulation. Preacidulation of muscles in carbonated Ringer-Locke solution had the same potentiating and weakening effects on humoral mediation of respiratory and non-respiratory muscles as did simultaneous acidification. For example, in figure 10, immersion of the rectus abdominis in bicarbonate-free Ringer-Locke solution containing a high concentration of carbon dioxide (between curves A and B) resulted in increased height and duration of contraction produced by subsequent flooding with a plain acetylcholine solution in curve 10B. Similar pre-acidulation of the frog sartorius muscle between curves 11A and B resulted in diminished contraction such as occurred with simultaneous acidification. It can be seen in these records that carbon dioxide alone had no stimulating effect.

Effect of lactic, phosphoric and hydrochloric acid on muscular contraction. Lactic, phosphoric and hydrochloric acids produced potentiation and weakening of contraction of the rectus abdominis and sartorius muscles respectively, similar to carbon dioxide (figs. 12, 13, 14). It was noted, however, that potentiation and weakening of the response of these muscles occurred only at relatively low pH values: 3.6 for lactic acid; 3.3 for phosphoric acid; 3.1 for hydrochloric acid. Even at these pH levels there was less potentiation than at pH 4.1 when carbon dioxide was used. Jacobs (1920) in describing the intracellular penetration of various acids, measured by color change of *Symphytum* flowers, states: "—it may be said briefly that of all the acids studied (carbonic, benzoic, salicylic, valeric, butyric, acetic, sulphuric and hydrochloric), carbonic is by far the most effective when pure solutions of *equal pH* are compared, in causing a visible change in intracellular acidity. For example, with a solution saturated with CO_2 at ordinary temperatures, a visible change in color usually begins in one or two minutes, while with the acids next in the order of their effectiveness (benzoic and valeric) fifteen to thirty minutes are required, with butyric, acetic and salicylic acids following in the order named. The mineral acids are only very slightly effective."

Effect of NaHCO_3 and combination of NaHCO_3 and CO_2 on contraction. Addition of sodium bicarbonate to an ambient acetylcholine solution resulted in an increased height and duration of contraction of the sartorius muscle of the frog (fig. 7D and 7F). Increased sodium bicarbonate content of the environment, however, caused a decrease in contraction of the frog's rectus abdominis and of the diaphragm of the alligator (fig. 1B and 16D), suggesting that the optimal environmental pH of the respiratory muscle fibers is on the acid side of neutrality. Maintenance of the external pH of a solution by the addition of enough sodium bicarbonate to buffer each increment of carbon dioxide so that the carbon dioxide tension was increased without any measurable pH change in the environment resulted in an increased height and duration of contraction

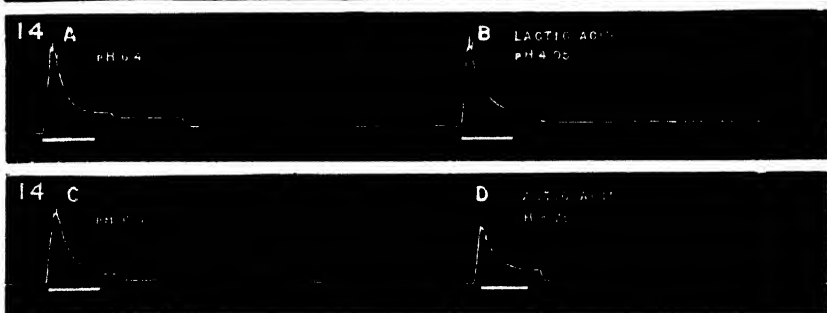
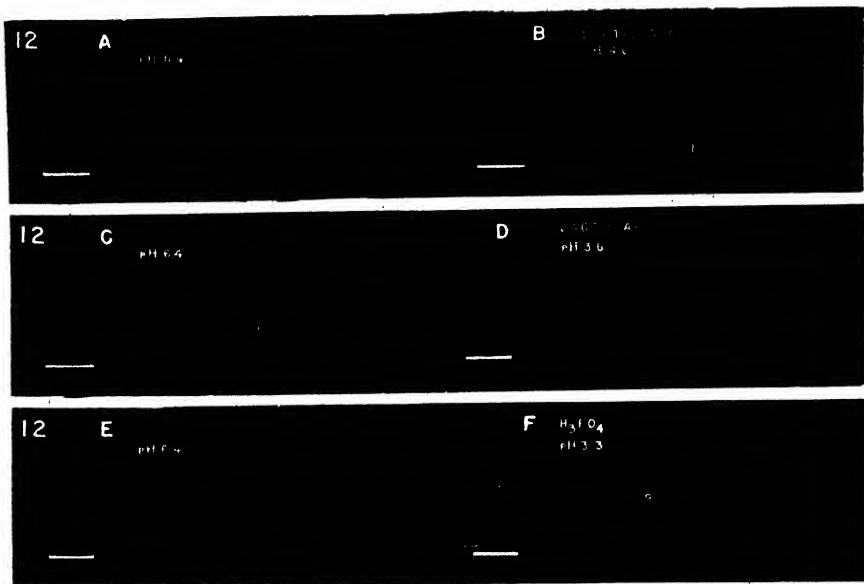


Fig. 12. Potentiation of response of the rectus abdominis muscle of the frog to acetylcholine by lactic and phosphoric acids. Addition of lactic acid to a bicarbonate-free Ringer-Locke solution sufficient to lower the pH to 4.0 had little or no potentiating effect, as shown in B. Further addition of lactic acid to pH 3.6 in D resulted in potentiation. Slight potentiation is shown in F in response to addition of H_3PO_4 to a bicarbonate-free solution sufficient to lower the pH to 3.3. A, C and E are control contractions in response to acetylcholine in bicarbonate-free solution. Note the slight potentiation by lactic and phosphoric acids in contrast to that shown by CO_2 in figure 1G at a higher pH. Acetylcholine 1-2,000,000.

Fig. 13. Effect of hydrochloric acid on response of the rectus abdominis muscle of the frog to acetylcholine (1-2,000,000). Note the slight potentiation in B, where HCl was added to a bicarbonate-free Ringer-Locke solution sufficient to lower the pH to 3.1, compared to the effect of CO_2 in figure 1G. A and C are control contractions in non-carbonated acetylcholine solutions.

Fig. 14. Effect of lactic acid on response of the sartorius muscle of the frog to acetylcholine (1-100,000). Addition of lactic acid to a bicarbonate-free Ringer-Locke solution sufficient to lower the pH to 4.05 had no effect upon contraction, as shown in B. Further addition of lactic acid to pH 3.75 in D, however, resulted in weakened contraction. A and C are control contractions in non-carbonated acetylcholine solutions.

of both types of muscles. (See fig. 15B and C for sartorius; fig. 1E for rectus abdominis; fig. 16B for diaphragm.) Judging from the work of Jacobs, an increase of intracellular cH probably occurred in these instances due to difference in penetration of carbon dioxide and sodium bicarbonate. Hartree and Hill (1924) believe that CO_2 is able to penetrate the cells of skeletal muscle and to raise their cH more quickly than other stronger acids. This gives a clue to the difference in response of respiratory and non-respiratory muscles to acid-base content of an acetylcholine solution.

A theoretical explanation of the differential effects of acid on respiratory and non-respiratory muscles. Had our experiments on acid-humoral mediation been confined to locomotor muscles the diminution of response to acetylcholine

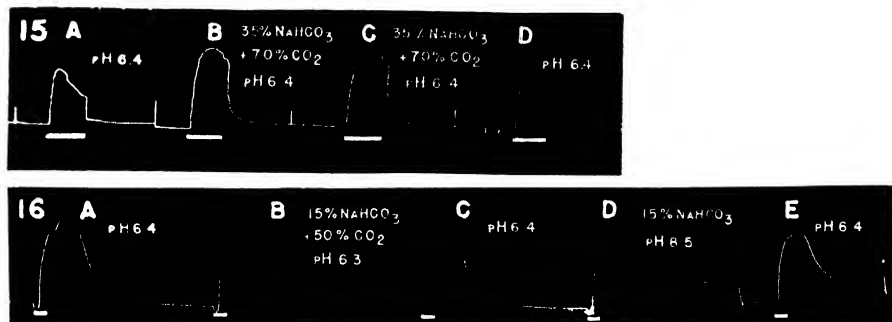


Fig. 15. Potentiation of response of the sartorius muscle of the frog to acetylcholine (1-100,000) by addition of 0.35 per cent NaHCO_3 and 70 volumes per cent CO_2 to a bicarbonate-free Ringer-Locke solution. A and D are control contractions in non-carbonated acetylcholine solutions. Since all the trials are at pH 6.4, higher pCO_2 presumably accounts for the potentiation in B and C (see discussion).

Fig. 16. Response of a strip of muscle from the diaphragm of an alligator to acetylcholine (1-100,000) in Ringer-Locke solution modified by NaHCO_3 and CO_2 . Potentiation of contraction produced by addition of 0.15 per cent NaHCO_3 and 50 volumes per cent CO_2 to a bicarbonate-free solution is shown in B. Weakening of response produced by the addition of 0.15 per cent NaHCO_3 to a bicarbonate-free solution is shown in D. A, C and E are control contractions in response to acetylcholine in a bicarbonate-free solution.

under the influence of acid might have stood as a conspicuous contradiction to potentiation common to the cholinergic systems; the heart, intestine, salivary glands, respiratory center and the central nervous system as a whole. Acid potentiation of contraction in respiratory muscles, however, precludes such a conclusion and calls for explanation instead since it hardly seems probable that the mechanism of neurohumoral mediation would differ basically in respiratory and non-respiratory muscles. The fact that potentiation of both respiratory and non-respiratory muscles can be produced by carbon dioxide under proper manipulation of the acid and base components of the environment upholds that contention. Why, then under ordinary procedures are the effects of acid on muscle so conflicting?

According to our concept of electrotonic stimulation of muscle (Gesell and

Hansen, 1945) two factors determine the effectiveness of the stimulation; *intensity* of the stimulus and the *excitability* of the structure subjected to the stimulus. Is it possible that intracellular and extracellular cH have selective and opposing effects as suggested by our experiments and those of Andrus (1924) on the rhythm of the heart of the cat? Andrus suggested that the "spontaneous rhythm is determined by the difference between the cH within and without the cell." He reasons as follows: "Within the physiological range it has been shown that the contents of the heart muscle cell are more acid than the fluid bathing it (Clark (3)). An increase of cH in the tissue fluid alone, by reducing the difference, leads to a slower development of the rhythmic excitation. When phosphoric acid is added to the solution surrounding the preparation, the cH of the bath tends to approach that of the cell, with consequent depression of the rate of excitation. With carbonic acid on the other hand, since the cH of the cell content rises as well, the effect of the same change in the external cH is less pronounced."

It has appeared to us that cH might influence physiological stimulation in several ways. 1. Increased intracellular cH would tend to increase the *intensity* of stimulation by increasing the amount of free intracellular acetylcholine. 2. Increased extracellular or membrane cH might oppose the electrotonic current flowing through and stimulating the membrane thereby diminishing the effects of free acetylcholine. 3. Increased extracellular or membrane cH might increase the threshold of stimulation which would also diminish the effects of acetylcholine. The feeble penetration of hydrochloric acid through the membrane of the muscle fiber plus an elevated extracellular cH would according to such reasoning meet the conditions for low potentiation of contraction noted in our experiments. Whatever the final answer may be, it is probable that the effects of cH in acid humoral mediation are multiple. It might be added that multiplicity of action would favor a specialized evolution of the acid-humoral mechanism required by specialization of function.

DISCUSSION. Carbon dioxide acting by itself in a simple Ringer-Locke solution showed no noticeable stimulating effects upon striated muscle. Only in conjunction with acetylcholine was its influence noticeable. It therefore does not function as a direct stimulant of muscle but as an indirect agent moderating the intensity of humoral stimulation. Evidence indicates that carbon dioxide works in a similar way upon the respiratory center. Endogenous acetylcholine liberated by synaptic bombardment is conserved by an intermediary action of carbon dioxide which indirectly controls the activity of the respiratory neurons and the volume of breathing (Gesell, Brassfield and Hamilton, 1942 and Gesell and Hansen, 1945). Thus diverse cholinergic systems have basic characteristics in common.

These basic similarities in diverse cholinergic systems (Brassfield and Gesell, 1942) have proven helpful in comparative considerations of other aspects of our problem—such as the specific action of carbon dioxide. As was pointed out above, carbon dioxide potentiates the stimulating action of acetylcholine on respiratory muscles at a higher pH level of the ambient solution than do lactic,

phosphoric and hydrochloric acids. Were it not for the demonstration of the comparatively high rate of diffusion of carbon dioxide across cellular membranes by Jacobs and Hartree and Hill the theoretical specific action of carbon dioxide in respiratory control would have demanded serious consideration. As it is, our experiments on muscle along with those of Jacobs on plant tissue indicate the critical importance of intracellular acidity. This concept of intracellular acidity has been held essential to an understanding of the chemical control of breathing since 1923 (Gesell, 1923). When this view was originally presented, the mechanism by which intracellular acidity played the critically determinant rôle in the control of breathing, however, was not clear. Facts only showed a correlation between intracellular acidity and pulmonary ventilation; but now that acetylcholine has been positively established as a neurohumoral stimulant (Loewi, Dale, Cannon and others) a simple and direct explanation is at hand. The acetylcholine liberated at the synapses of the respiratory neuron stimulates the respiratory center. The cholinesterase existing in greatest concentration at the site of liberation of acetylcholine controls the rate of destruction of the humor. If acetylcholine is the great integrator that accumulated evidence implies, the close physical and chemical association of acetylcholine and cholinesterase combined with their intracellular site of function would seem finally to explain why intracellular acidity is the factor which determines the activity of the respiratory center.

Were it not for the hyperpnea of acidosis such as occurs in diabetes and for the increased breathing resulting from oxygen lack, the specific action of carbon dioxide might also warrant more consideration. As is well known, the intensity of breathing increases progressively as the amount of the respiratory regulator, CO_2 , diminishes. Until the theory of specificity of action of carbon dioxide attempts to meet this paradoxical stimulation and so long as facts and observations give evidence that the cH theory of respiratory control meets conditions imposed upon it the invocation of an intangible and contradictory mechanism such as specificity of action of carbon dioxide cannot be accepted as an explanation.

Since buffer base is steadily reduced by the stronger acids which are continually formed in diabetic acidosis, the tissues gradually lose their power to buffer carbon dioxide; thus carbon dioxide which is formed in the body becomes increasingly effective as an *acid*. As acidosis advances, the respiratory center, therefore, turns more acid; endogenous acetylcholine liberated at the respiratory neurons is more effectively conserved; and the center is more intensely stimulated. Carbon dioxide is consequently eliminated in greater quantity and the concentration of carbon dioxide falls throughout the body. The respiratory center nevertheless continues super-acid and therefore superactive, since it, like all the tissues of the body, is poorly buffered against its own metabolic carbon dioxide and that of the tissues in general. To the best of our knowledge increasing effects of CO_2 and a simultaneous diminution of its concentration is a combination unique to the cH mechanism of respiratory control.

The explanation of the paradoxical hyperpnea of oxygen lack, that is, increas-

ing ventilation with a concomitant decreasing $p\text{CO}_2$, is somewhat longer and in the interest of clarity is presented in 9 distinct steps each of which rests upon experimental evidence (Gesell, 1929, 1940; Bernthal, 1944, and Gesell and Hansen, 1945). 1. A localized acidosis develops in the chemoceptors which are the chemically sensitive outposts protecting the body against oxygen lack (Heymans). 2. This local acidosis is due to excessive formation of lactic acid and thus resembles in all essential details the generalized acidosis of diabetes. 3. The chemoceptors belong to the cholinergic system. They are highly sensitive to acetylcholine and acid, and are therefore activated by the local acidosis resulting from the lack of oxygen. 4. The consequent increased bombardment of the respiratory neurons and the correspondingly augmented liberation of endogenous acetylcholine increases the activity of the respiratory center. 5. The resulting increased elimination of carbon dioxide tends to lower the cH of the entire body including the respiratory center. 6. Lowered cH of the respiratory center results in a greater destruction of endogenous acetylcholine liberated at the center. 7. The chemoceptors, due to a continued local formation of anaerobic acids, however, remain superactive. 8. This superactivity sustains a super-bombardment of the respiratory center. Sustained super-liberation of acetylcholine at the respiratory neurons more than compensates for the greater destruction of acetylcholine resulting from a lowered cH of the center. 9. The center, therefore, continues superactive despite a lowering of the carbon dioxide content of the tissues.

Banus, Corman, Perlo and Popkin (1944) have recently studied the compensation of anesthetized dogs to increasing acidosis due to intravenous injections of hydrochloric and lactic acids. These studies are of particular interest because the authors eliminated the chemoceptor reflexes and thus focussed attention on the effects of acid on the respiratory center. They conclude as follows:

Results in all experiments were similar. The existing acidosis, as shown by the lowering of the bicarbonate concentration of arterial blood was compensated for by increased ventilation and a consequent lowering of the CO_2 tension of arterial blood, the latter being in inverse proportion to the volume of ventilation.

The compensation may be efficient enough to maintain the pH of arterial blood constant at least within ± 0.01 . As the chemoceptors in the experimental animals were ineffective, we will have to accept the respiratory center as the regulatory mechanism unless we invoke some other unknown mechanism, and therefore the respiratory center must be sensitive to changes in hydrogen ion concentration at least as small as pH 0.01.

The findings of Banus et al. are interesting in several respects. First of all the experimental conditions are relatively simple in that the chemoceptors are denervated. Since acidosis in parts of the body other than the chemoceptors is not known to increase the synaptic bombardment of the center, the amount of endogenous acetylcholine liberated at the respiratory center would remain relatively uniform. That confines the effects of acid to the respiratory center and warrants a comparison of respiratory activity with that of our simple isolated respiratory muscle subjected to an acidified acetylcholine solution.

It is relevant to recall that acidosis produced by injection of hydrochloric

acid is similar in essential details to that of diabetic acidosis. The tissues undoubtedly lose their power to buffer carbon dioxide as proven by the migration of base into the blood. Late injections of acid produce relatively smaller reductions in the sodium bicarbonate content of the blood than the first injections. Bearing in mind this movement of bicarbonate across cellular membranes it becomes clear why the injection of sodium bicarbonate in diabetic acidosis allays hyperpnea despite an increase of alveolar $p\text{CO}_2$. The respiratory center is now better buffered and maintains a relatively uniform pH at higher $p\text{CO}_2$. It therefore would seem from all evidence available that acidosis in *unanesthetized* man increases breathing in a manner indistinguishable from that of experimental acidosis in the *anesthetized* dog. An unsupported interpretation of hyperpnea of acidosis in terms of anesthesia can lead only to an unfortunate confusion in a most important phase of experimental physiology and clinical medicine which experimentally seems to be resolving itself into simple basic principles of neurophysiology.

Another point raised by our experiments is the possible biological significance of the differential response of respiratory and non-respiratory muscles. In an earlier paper (Gesell and Hansen, 1945) it was suggested that acid-humoral mediation is a very primitive mechanism of nervous integration which served originally to provide only for the simple but vital need of oxidative energy. Once acquired and perfected for that use it was too valuable to discard. Retained as a basic mechanism of nervous integration it was in turn necessary that humoral mediation be molded to conform with the evolutionary requirements of higher nervous integrations. That the acid-humoral mechanism was retained is evidenced by the fact that all parts of the central nervous system are sensitive to both humor and acid. That the acid-humoral mechanism was molded to fit specialized requirements is indicated by the differences of sensitivity to acid at various stations in the central nervous system.

It is therefore tentatively suggested that the differential response of respiratory and non-respiratory muscles represent a peripheral molding of function to fit the metabolic needs of the organism. Sensitization of respiratory muscles to acetylcholine by acid could function as an accessory mechanism for the provision of oxygen especially in the lower forms of life. On the other hand a similar sensitization of the great mass of locomotor muscles could only serve to increase the energy requirements of the body without contributing to the supply of oxygen. In conclusion, it may therefore be suggested that carbon dioxide acts as an easily diffusible acid which increases the intracellular acidity in the region of the motor end plate of skeletal muscle. This prolongs and increases the stimulating effect of acetylcholine at its normal site of liberation. In respiratory muscle this mechanism effects an increase in height and strength of contraction, but in locomotor muscles an additional unknown effect of acid decreases the height and strength of contraction. It is proposed that this difference in response to acidity may serve as a protective mechanism against excessive locomotor activity when the emergency is purely respiratory.

SUMMARY

The response of respiratory and of locomotor muscles of the frog, turtle and alligator to exogenous acetylcholine was compared under varying conditions of acid-base equilibrium.

Muscles associated with the act of breathing (rectus abdominis, geniohyoid and mylohyoid of the frog, mylohyoid of the turtle and diaphragm of the alligator) responded more actively to acetylcholine when acids were added to the ambient Ringer-Locke solution.

Muscles which are primarily locomotor in function (sartorius, peroneus and gracilis of the frog) were less responsive to acetylcholine when acids were added to the solution.

Carbon dioxide, lactic, phosphoric and hydrochloric acids produced qualitatively similar results. This is in agreement with the qualitatively similar effects which these acids produce upon respiration. It was suggested that the motor and respiratory responses are comparable neurohumoral phenomena and that the common denominator accounting for the similarity of action of widely differing acids is cH . Quantitatively the effects of these acids vary in the order of their enumeration. The well known high rate of diffusion of carbon dioxide through cellular membranes is thought to explain why it is so predominantly effective. This conclusion implies the importance of intracellular acidity in humoral mediation as opposed to the unsupported hypothesis of specificity of action of carbon dioxide in control of breathing.

When the ambient solution was modified by the addition of carbon dioxide and sodium bicarbonate in proportions which maintained a uniform environmental pH the response of the locomotor muscles to acetylcholine was found to be increased. Since carbon dioxide moves so freely this observation is thought to indicate that acid, under properly controlled conditions, is capable of potentiating humoral mediation in locomotor as well as respiratory muscles. It is suggested that the potentiation under these conditions is a result of an increased intracellular cH .

This conclusion leads to the suggestion that an increasing environmental cH in some way opposes humoral mediation in locomotor muscles. (See discussion.)

It was suggested that the differential effect of acid on respiratory and locomotor muscles may be the result of an evolutionary adjustment which serves to avoid unnecessary locomotor activity during emergencies primarily respiratory in nature.

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THE BENEFICIAL EFFECTS OF YEAST ON THE CARDIAC FAILURE OF HYPERTHYROID RATS

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That hyperthyroidism results in an increased vitamin B requirement has been demonstrated by a number of investigators. Administration of thyroxin or thyroid gland to adult rats results in a prompt loss of weight preventable by administration of yeast or other concentrates of vitamin B (1, 2). In 1938 Drill and associates initiated a series of experiments on the effects of vitamin B₁ and yeast on adult hyperthyroid rats that had lost weight. It was observed that vitamin B₁ alone prevented further loss in weight, but that rats did not regain their initial weight unless a rich source of the vitamin B complex was also supplied (2, 3). Subsequent work has demonstrated that pyridoxine and calcium pantothenate could effectively replace the vitamin B complex in the diet of the hyperthyroid rat (4). Thus in addition to vitamin B₁ both pyridoxine and pantothenic acid are required in larger amounts during experimental hyperthyroidism in the rat.

The present experiment was undertaken in an effort to determine the effects of thyroid feeding on dietary requirements for other components of the vitamin B complex. Rats can be raised to maturity on purified rations containing synthetic B complex factors (thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, calcium pantothenate and choline chloride) (5-7). On such a ration the intestinal flora or the animal's own tissues synthesize sufficient quantities of nicotinic acid, biotin, inositol, para-aminobenzoic acid, folic acid and presumably other as yet unidentified members of the vitamin B complex to meet the requirements for growth. If the requirements for any of the above factors are increased during thyroid feeding above the level synthesized by the organism or its intestinal flora, animals maintained on such rations supplemented with thyroid should develop pathological manifestations preventable by administration in suitable quantity of the missing factor(s).

PROCEDURE AND RESULTS. Three basal diets were employed in the present experiment: diets A, B and C. Diets A and B were purified rations containing the B complex factors in synthetic form and differing only in the level at which these vitamins were administered. Diet C was similar in composition but contained yeast in place of the synthetic B factors. All three rations were supplemented with 0.0, 0.5 and 1.0 per cent thyroid respectively.

Sixty female rats of the Long-Evans strain were employed in the present experiment. At twenty-three days of age litter mates were divided as far as possible among the nine experimental groups listed in table 1. Animals were kept in metal cages with screen bottoms to prevent access to feces, and sufficient food was administered to assure *ad lib* feeding. Feeding was continued for sixty days.

Growth, gross appearance and length of survival. A significant difference in length of survival was observed in thyroid-fed rats on the various diets employed. Animals on yeast-containing rations lived significantly longer at given levels of thyroid than litter mates on synthetic rations (table 2). No significant differences were noted between thyroid-fed rats on diets A and B or between animals receiving 0.5 and 1.0 per cent thyroid. Growth was retarded on all thyroid-containing diets; but animals grew consistently on these rations, and no plateauing or loss of weight occurred before death. In all dietary groups thyroid-fed rats developed some degree of diarrhea which persisted for the first month of

TABLE 1
Composition of experimental diets and distribution of rats

	A1	A2	A3	B1	B2	B3	C1	C2	C3
Thyroid*	0	0.5	1.0	0	0.5	1.0	0	0.5	1.0
Yeast†	0	0	0	0	0	0	12.0	12.0	12.0
Vitamin test casein‡	22.0	22.0	22.0	22.0	22.0	22.0	22.0	22.0	22.0
Salt mixtures§	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Sucrose	73.5	73.0	72.5	73.5	73.0	72.5	61.5	61.0	60.5
Distribution of rats	6	7	8	6	8	7	6	6	6

To each kilogram of diet A were added the following synthetic vitamins: thiamine hydrochloride 2 mgm., riboflavin 4 mgm., pyridoxine hydrochloride 2 mgm., calcium pantothenate 30 mgm., choline chloride 1200 mgm. and 2 mgm. 2-methylnapthaquinone.

To each kilogram of diet B were added: thiamine hydrochloride 80 mgm., riboflavin 40 mgm., pyridoxine hydrochloride 20 mgm., calcium pantothenate 200 mgm., choline chloride 1200 mgm. and 5 mgm. 2-methyl-napthaquinone.

Each rat on diets A, B and C also received the following daily supplement: corn oil (Mazola) 500 mgm., and a vitamin A-D concentrate¶ containing 50 U.S.P. units of vitamin A and 5 U.S.P. units of vitamin D.

* U.S.P. desiccated thyroid, Armour and Co., Los Angeles, California.

† Brewers' type yeast no. 200, Anheuser-Busch, Inc., St. Louis, Mo. Each gram according to the manufacturer contained the following vitamin potencies: thiamin 600 micrograms, riboflavin 75 micrograms, pyridoxine 100-125 micrograms, pantothenic acid 420-560 micrograms, nicotinic acid 350-500 micrograms and biotin 0.2-0.5 microgram.

‡ Vitamin test casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

§ Salt mixture no. 1 (Sure, B. [8]).

¶ Nopco fish oil concentrate, assaying 800,000 U.S.P. units of vitamin A and 80,000 U.S.P. units of vitamin D per gram.

feeding; and in all instances their fur became dull, rough and dirty. After three weeks of feeding alopecia was noted in approximately one-third of the thyroid-fed rats, irrespective of the basal ration or the level of thyroid employed. Loss of fur was particularly marked around the eyes and the dorsal surface of the neck and upper back. No relationship was observed however between the degree of alopecia and length of survival.¹ Animals from whose diet thyroid was withheld were free of diarrhea or alopecia, and fur remained clean and sleek.

The immediate cause of death was apparently cardiac failure. Animals

¹Subsequent to the sixth week of feeding new hair replaced the areas of alopecia.

that appeared well nourished, were gaining weight and differed from litter mates not receiving thyroid only in lower body weight, various degrees of alopecia and the appearance of their fur, would suddenly develop symptoms of central nervous system anoxia followed by death in a matter of a few minutes.² In one instance where autopsy was performed at the first evidence of approaching death, the heart was observed in fibrillation. Invariably the ventricles were found at autopsy in a state of contraction, with the auricles and pulmonary

TABLE 2
Effects of thyroid feeding on growth and length of survival of the immature female rat

DIETARY GROUP	PER CENT THYROID	NUMBER OF ANIMALS	INITIAL WEIGHT	BODY WEIGHT ON 24TH DAY OF EXPERIMENT*	BODY WEIGHT ON 60TH DAY OF EXPERIMENT*	PER CENT SURVIVING†	AVERAGE LENGTH OF SURVIVAL OF DECEASED*
			<i>grams</i>	<i>grams</i>	<i>grams</i>		<i>days</i>
A1	0	6	45.8	111.0 ± 6.6 (6)	183.2 ± 10.7 (6)	100	
A2	0.5	7	46.5	96.7 ± 5.3 (4)	130.0 (1)	14.3	26.5 ± 3.3
A3	1.0	8	48.6	90.0 (1)		0	21.3 ± 1.5
B1	0	6	45.2	104.2 ± 6.8 (6)	173.2 ± 11.0 (6)	100	
B2	0.5	8	44.5	83.7 ± 0.7 (3)		0	23.9 ± 1.0
B3	1.0	7	47.0	75.7 ± 4.8 (3)		0	24.0 ± 1.2
C1	0	6	45.0	113.5 ± 5.5 (6)	165.2 ± 8.9 (6)	100	
C2	0.5	6	45.0	89.5 ± 4.7 (6)	138.7 ± 15.3 (3)	50	45.0 ± 6.3
C3	1.0	6	44.8	85.7 ± 2.8 (3)	122.0 (1)	16.7	36.2 ± 7.3

The values in parentheses indicate the number of animals which survived of which this is an average.

* Including standard error of the mean calculated as follows: $\sqrt{\frac{s^2}{n}} / \sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

† Experimental period 60 days.

vein engorged with blood. Some degree of congestion was present in the liver, but with the exception of enlarged kidneys, infantile ovaries and a hypertrophied ventricular wall, the gross appearance was essentially that of a well nourished normal rat. It might be added that approximately sixty per cent of the deaths occurred while animals were removed from their cages, either during cleaning or while having their electrocardiograms taken. As far as possible however experimental conditions were kept constant for all rats.

²In some instances animals survived for several hours, but the majority died within ten minutes.

Electrocardiographic findings. The sudden deaths of animals receiving thyroid suggested that cardiac failure was the immediate cause of death. Electrocardiograms accordingly were taken in an effort to determine the degree of myocardial involvement.³ A Cambridge electrocardiograph was employed, resistance standardized for each animal, and electrocardiograms taken on unanesthetized rats on the 26th, 30th and 59th days of feeding. The standard three leads were taken of all animals surviving at the time and occasional tracings of lead IV. The most consistent response to thyroid feeding was an increased ventricular rate. On thyroid-free rations (A1, B1 and C1) the heart rate averaged 545 beats per minute (range 510 to 600) and 520 per minute (range 480 to 540) after 30 and 59 days of feeding respectively. On diets containing thyroid the heart rate ranged from 620 to 690 (average 660) and from 660 to 720 (average 690) per minute after 30 and 59 days respectively on the dietary regime. No significant differences were observed between the 0.5 and 1.0 per cent thyroid series or between animals on the synthetic or yeast-containing rations.

Additional abnormalities were observed in the electrocardiographic complexes, increasing in severity with the duration of thyroid feeding. In the thirty day series the main abnormalities in the electrocardiogram of the thyroid-fed rat were: 1, an increased amplitude of the P wave; 2, increased amplitude of the main deflections (R or S waves); 3, increased amplitude of the T wave and 4, elevated ST interval in leads II and III. By the 59th day of feeding the P waves had increased further in amplitude, the ST segment was either depressed or elevated in leads II, III and IV, the PR interval was increased, left ventricular predominance was observed as were inverted T_s, ventricular extra systoles and occasional dropped beats. The R wave was notched in lead IV. These changes were not uniformly present in all thyroid-fed rats, but they were absent from the electrocardiogram of all animals from whose diet thyroid was withheld. No significant differences were observed between the 0.5 and 1.0 per cent thyroid series or between animals on the synthetic or yeast-containing rations. It is apparent therefore that the prophylactic properties of yeast noted in the increased survival time of thyroid-fed rats were not manifest in electrocardiographic tracings.

*Gross and histological findings.*⁴ As soon as possible after death or at autopsy on the sixtieth day of feeding, hearts were weighed, fixed in formol and sections prepared stained with hematoxylin and eosin. In the yeast series hearts averaged after sixty days of feeding 788.6 mgm., 1133.2 mgm. and 1277 mgm., for animals receiving 0.0, 0.5 and 1.0 per cent thyroid respectively. Similar values were obtained for rats on synthetic diets that survived the experimental period. Histologically the heart of thyroid-fed rats resembled those described by Hashi-

³We wish to express our sincere appreciation to Dr. Douglas R. Drury of the Department of Physiology, University of Southern California, for his interest and assistance with the electrocardiograms.

⁴We are indebted to Dr. Ernest M. Hall, Professor of Pathology, University of Southern California Medical School, for the examination and description of the histological material.

moto (9) in animals dying during the course of thyroid feeding. The main abnormalities consisted of a number of small scars with loss of muscle fibers and histiocytic cellular infiltration. The histiocytes were particularly numerous in the neighborhood of blood vessels, and foci of closely packed histiocytic cells were abundant at the site of fiber dissolution, in the scars and throughout the interstitial tissue. Many scars ran lengthwise in the muscle fibers and in some areas one-third to one-half of the fibers were destroyed. Animals on thyroid-free rations did not exhibit the above pathology. The above findings were observed in all thyroid-fed rats; in the synthetic series however additional pathology was noted. Zenker's degeneration of muscle fibers was marked with actual necrosis in some areas and calcification of degenerating fibers. Moderate degrees of fibrous thickening and cellularity were observed in the valves, especially the distal third; and on occasion the epicardium was thickened and edematous. The number of animals in each group was too small to permit a statistical evaluation of the above findings; it might be pointed out however that the most severely affected animals were invariably those maintained on synthetic rations.

DISCUSSION. This is the first report to our knowledge concerning the effects of thyroid feeding to animals maintained on synthetic rations. Results indicate that rats on such diets are extremely sensitive to thyroid feeding, with sensitivity manifesting itself in sudden death apparently due to cardiac failure. Length of survival was markedly prolonged in thyroid-fed rats receiving their vitamin B complex in the form of yeast. The protective properties of yeast were not due to any of the B vitamins present in the synthetic rations. This is indicated by the fact that the length of survival and incidence of mortality in the High Vitamin Series (B2 and B3) did not differ significantly from that observed in the Low Vitamin Series (A2 and A3) although it contained 40 times as much thiamine, 10 times as much riboflavin, 10 times as much pyridoxine and $6\frac{2}{3}$ times as much calcium pantothenate as was present in the latter. Furthermore the amount of these vitamins in the High Vitamin Series was greater than that present in the yeast-containing diets (C2 and C3) although no prolongation of survival time was observed in the former groups. The increased survival of thyroid-fed rats on yeast-containing rations suggests therefore that some factor was present in yeast other than the vitamins indicated above that prolonged survival in thyroid-fed rats under the conditions of the present experiment.

In addition to thiamine, riboflavin, pyridoxine, choline and pantothenic acid, yeast contains significant quantities of nicotinic acid, inositol, para-aminobenzoic acid, biotin, folic acid and presumably other as yet unidentified vitamins in addition to protein and ash. In the absence of thyroid rats on synthetic rations A1 and B1 synthesized sufficient quantities of these factors to meet the requirements for growth—and reproduction as well (7). The addition of thyroid however resulted in a number of early deaths—although length of survival was significantly prolonged if yeast were administered in place of the synthetic B vitamins. These findings suggest that nutritional deficiencies induced by thyroid feeding were responsible for the early deaths. It is not unlikely that thyroid feeding interfered with the synthesis of essential nutrients

by the organism or its intestinal flora or that thyroid increased the requirements for nutrients present in yeast but not present in sufficient quantity in the synthetic diets employed or synthesized in sufficient amounts by the intestinal flora or the animal's tissues.

That thyroid feeding will induce cardiac lesions in experimental animals has been demonstrated by a number of investigators (9-13). Similar changes however have been observed following administration of adrenalin (14-17) and in other conditions resulting in accumulations of adrenalin and adrenalin-like substances (catechol derivatives) in heart muscle (18, 19). These findings suggest that cardiac pathology in thyroid-fed rats may be due at least in part to myocardial accumulations of adrenalin and adrenalin-like substances. Raab (19) has recently reviewed the relationship of these substances to manifestations of cardiac pathology. Such effects as cardiac hypertrophy and dilatation, anoxic changes of the electrocardiogram, myocardial degeneration, heart failure and eventually cardiac death may be caused by high concentrations of adrenalin and adrenalin-like substances in heart muscle and are not necessarily primary effects of cardiovascular pathology. Of particular importance in this regard is the marked sensitivity of the hyperthyroid organism to adrenalin. Raab for example has demonstrated that doses of adrenalin relatively innocuous for the normal rat proved fatal within three to five minutes when injected into animals that had been pretreated with thyroxin for three days. Similarly the concentration of adrenalin and adrenalin-like substances in heart muscle following an injection of adrenalin was markedly increased if the animal were pretreated with thyroid (19).

No data are available to account for the increased survival time of thyroid-fed rats on yeast-containing rations. Raab and Supplee (20) however observed a striking increase in the concentration of adrenalin and adrenalin-like substances in the heart muscle of vitamin B₁ deficient rats, with prompt return to normal following administration of thiamine hydrochloride. Other nutrients may similarly be involved. The present experiment indicates that some factor in yeast prolonged survival in thyroid-fed rats under the conditions of this experiment. Further data on the effects of para-aminobenzoic acid, inositol, biotin, folic acid, nicotinic acid and various yeast fractions in prolonging survival of thyroid-fed rats on synthetic rations, and the possible effect of these factors on the concentration of adrenalin and its catechols in heart muscle should provide valuable information as to the active ingredient involved.

SUMMARY

Female rats were placed at weaning on purified rations containing 0.0, 0.5 and 1.0 per cent desiccated thyroid. Three basal rations were employed. In two of the rations B vitamins were administered as synthetic factors; in the third they were present as yeast.

Rats maintained on synthetic rations were extremely sensitive to thyroid feeding, with sensitivity manifesting itself in sudden death apparently due to cardiac failure. Length of survival was markedly prolonged in thyroid-fed rats

receiving their vitamin B complex in the form of yeast. No pathology was observed on thyroid-free rations.

Growth was retarded in all thyroid-fed rats but no plateauing or loss of weight occurred before death. Alopecia was noted subsequent to the third week of thyroid feeding, particularly around the eyes and the dorsal surface of the neck. No relationship was observed however between the degree of alopecia and length of survival.

Abnormalities were noted in the electrocardiogram of all thyroid-fed rats. These consist of an increased ventricular rate, increased amplitude of the P, R or S and T waves, depressed or elevated ST segments, inverted T waves, ventricular extra systoles, occasional dropped beats, increased PR interval and left ventricular predominance. No significant differences were observed between rats on the synthetic and yeast-containing rations.

Cardiac pathology was observed in all thyroid-fed rats. In addition to enlargement of the heart the following abnormalities were observed: numerous scars with loss of muscle fibers and histiocytic cellular infiltration, particularly marked in the neighborhood of blood vessels and the site of fiber dissolution; Zenker's degeneration of muscle fibers, necrosis and calcification of degenerating fibers; and hypertrophy of endothelial cells. The most severely affected animals were those maintained on synthetic rations.

The suggestion is made that some factor is present in yeast other than the vitamins present in the synthetic rations that prolonged survival in thyroid-fed rats. The possible relationship of this factor to the concentration of adrenalin and adrenalin-like substances in heart muscle during thyroid feeding is discussed.

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GROWTH AND HEMOGLOBIN PRODUCTION IN DOGS ON PURIFIED RATIONS¹

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Under our experimental conditions a purified ration containing sucrose, casein, cottonseed oil and salts and supplemented with the fat soluble vitamins A and D and the water soluble vitamins thiamin, riboflavin, nicotinic acid, pyridoxine, pantothenic acid and choline, has been found to be remarkably satisfactory for growing dogs. In fact excellent growth and blood regeneration have been obtained even when these rations contained levels of succinylsulfathiazole as high as 4 per cent (1). However, recent reports by Lambooy and Nasset (2) and Smith (3, 4) indicate that additional factors are necessary for optimum growth, maintenance of a healthy skin and prevention of anemia. Since the basal rations used by these workers differed somewhat from ours, the following experiments were undertaken.

METHODS. Three litters of recently weaned mongrel dogs were treated twice with an anthelmintic (tetrachlorethylene), freed of external parasites with rotenone, and placed in individual cages. The floors of the cages were of concrete and covered with clean wood shavings. All animals were fed *ad libitum* and watered daily, and received an aqueous suspension of the B vitamins twice a week administered by pipette. Haliver oil and Drisdol furnished adequate amounts of vitamins A and D.

Three milliliter blood samples were obtained from the radial vein on the same day every week before the morning feeding, and hemoglobin, red blood cell and hematocrit values were determined by methods previously described (5).

The dogs were divided into four groups: group I (2 dogs) received ration I which is our basal ration, group II (3 dogs) was fed ration II which is the same as ration I except for the addition of p-aminobenzoic acid and inositol, group III (2 dogs) was given ration III which is a high fat ration comparable to that of Smith, and group IV (4 dogs) was fed ration IV which contains alcohol extracted casein. Table 1 gives the composition of the different rations.

RESULTS. Dog 1, a littermate of dogs 3 and 4 in group II, received basal ration I for a period of three months and dog 2, a littermate of dogs 8 to 11 in group IV was kept on the basal ration I for ten months. Throughout the course of the experiment both dogs grew rapidly and maintained a healthy appearance.

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However, after five months on the basal ration, dog 2 which chanced to be a black dog, became slightly gray and remained so throughout the experiment. The hemoglobin curve for this animal was seen to plateau at 12 to 14 grams per cent (fig. 1). Dog 1 showed a similar hemoglobin curve even though kept on the experiment for a shorter period of time.

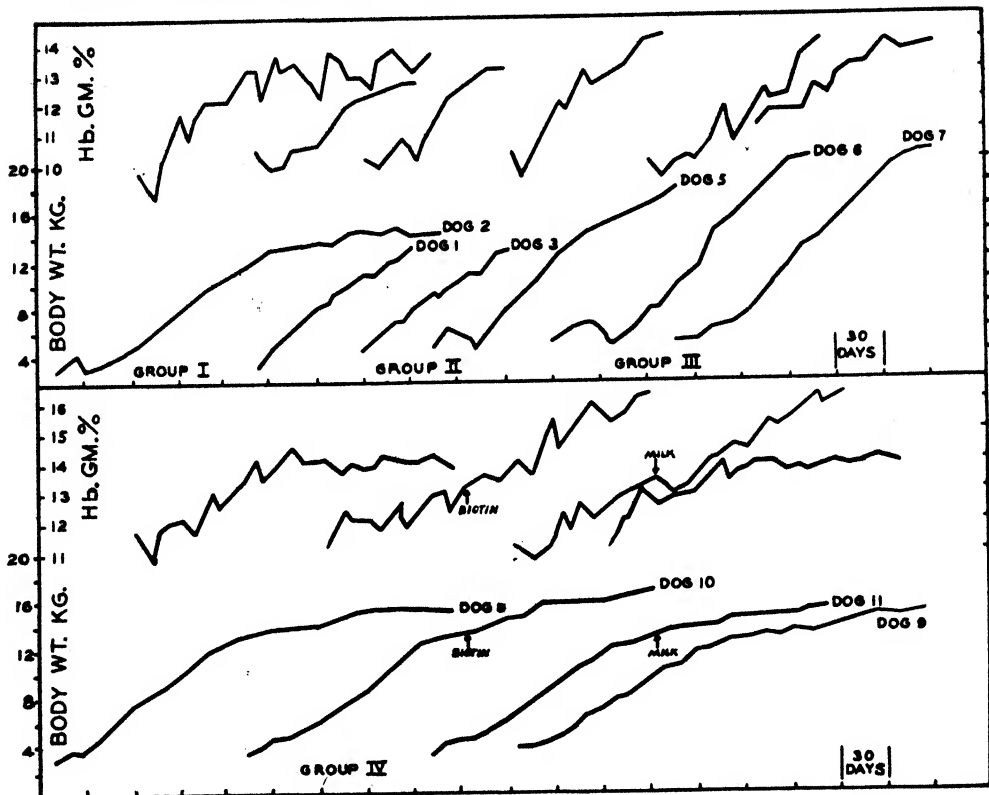


Fig. 1. Growth and hemoglobin curves for dogs 1 to 11.

Group I (dogs 1 and 2) received ration I, group II (dogs 3 and 5) were kept on ration II, group III (dogs 6 and 7) were fed ration III and group IV (dogs 8, 9, 10 and 11) received ration IV plus supplements where indicated. Dogs 8, 9, 10 and 11 were littermates of dog 2, dogs 1 and 3 were littermates and dogs 6 and 7 were littermates of dog 5.

Ration II was fed to dogs 3 and 4 for four months while dog 5, a littermate of dogs 6 and 7, received ration II for five months. Throughout the experimental period dogs 3 and 4 compared favorably in all respects with their littermate control (dog 1) which received ration I. Likewise dog 5 showed good growth, a normal appetite and a rising hemoglobin curve which reached a maximum of 14 grams per cent at the conclusion of the experiment (fig. 1). Therefore the addition of p-aminobenzoic acid and inositol brought about neither detrimental nor beneficial results.

Excellent growth was obtained with dogs 6 and 7 which were maintained on the high fat ration III for a period of five months. During this time the dogs averaged 400 to 500 grams of food per day and the weekly weight gain approximated 1 kgm. The hair coat on these animals became quite lustrous and the skin lesions observed by other workers failed to appear.

Dogs 8, 9, 10 and 11 were placed on the purified casein ration IV and kept on experiment for nine months. At the end of the second month varying degrees of grayness were noted in the black dogs, and at the end of the fifth month the hemoglobin curves showed a plateau at 13 to 14 grams per cent (fig. 1). Since these hemoglobin levels appeared to be suboptimal, one dog was placed on a mineralized milk diet (6), supplemented with vitamins as in ration IV. Dog

TABLE 1
Composition of the various rations used

	RATIONS I AND IV*	RATION II	RATION III
Sucrose.....	66%	66%	36%
Casein.....	19%	19%	40%
Cottonseed oil...	11%	11%	18%
Cod liver oil....	0%	0%	2%
Salts.....	4%	4%	4%
Thiamin.....	0.100 mgm./kgm./day	0.100 mgm./kgm./day	0.100 mgm./kgm./day
Riboflavin.....	0.100 mgm./kgm./day	0.100 mgm./kgm./day	0.100 mgm./kgm./day
Nicotinic acid...	2 mgm./kgm./day	2 mgm./kgm./day	2 mgm./kgm./day
Ca-d-pantothene.....	0.500 mgm./kgm./day	0.500 mgm./kgm./day	0.500 mgm./kgm./day
Pyridoxine.....	0.060 mgm./kgm./day	0.060 mgm./kgm./day	0.060 mgm./kgm./day
Choline chloride...	50 mgm./kgm./day	50 mgm./kgm./day	50 mgm./kgm./day
Inositol.....	0	0.500 mgm./kgm./day	0
p-aminobenzoic acid.....	0	0.500 mgm./kgm./day	0
Vitamin A.....	1800 I.U./dog/day	1800 I.U./dog/day	1800 I.U./dog/day
Vitamin D.....	500 I.U./dog/day	500 I.U./dog/day	500 I.U./dog/day

* Ration IV contains alcohol extracted casein in place of the acid washed casein of ration I.

11 therefore received three liters of mineralized milk per day for three and one-half months, while dogs 8 and 9 remained on the purified casein ration as controls. One week later dog 10 was given biotin supplements of 5 gamma per kilogram of body weight per day. At the conclusion of the experiment the hemoglobin level for dog 11 had increased to a value of 16.5 grams per cent and that of dog 10 had reached a value of 16 grams per cent. The control animals 8 and 9, however, had remained at a level of 14 grams per cent (fig. 1). No significant differences could be found in growth rates.

In order to study these changes further, another litter of six dogs was placed on experiment. These animals were fed ration IV *ad libitum* and were placed in cages with raised wire bottoms to prevent coprophagy. By the end of the second month, all dogs had developed achromotrichia. A plateau in hemo-

globin curves again occurred at about the fifth month at which time the six dogs were divided into three groups. Two dogs remained on the purified casein ration and served as controls, two other animals received supplements of 5 gamma of biotin per kilogram of body weight per day and the remaining two dogs received fresh liver at a level of 15 grams per 100 grams of ration consumed per day. Food consumption remained constant throughout, but dogs 14 and 15, which received the liver supplement, showed a marked weight increase. Hemoglobin values failed to increase, however, even though appreciable amounts of biotin are present in fresh liver. Dogs 16 and 17, which received biotin supplements, showed a marked response in hemoglobin and, to some extent, a weight increase. Dogs 12 and 13 which served as controls continued to demonstrate relatively low hemoglobin levels of 11 to 13 grams per cent.

DISCUSSION. The results obtained in this experiment indicate that under experimental conditions such as prevailed, dogs will grow well and maintain a good state of health without receiving any B vitamins other than thiamin, riboflavin, pyridoxine, pantothenic acid, nicotinic acid and choline. However, when a highly purified alcohol extracted casein ration is fed, hemoglobin will plateau at 11 to 14 grams per cent and severe achromotrichia often develops. These data, although not conclusive, indicate that biotin is necessary for the attainment of hemoglobin values greater than 14 grams per cent. This belief is further substantiated by the results obtained with dog 11 which received the mineralized-vitaminized milk ration. According to Hodson (7), fresh milk contains approximately 50 gamma of biotin per liter, and since this animal received 3 liters of milk per day, the intake of biotin is sufficiently high to account for the increased hemoglobin production. However, in contrast to this, dogs 14 and 15 which received fresh liver did not demonstrate this hemoglobin response over their controls, but did show an increased growth rate. Based on food consumption, the amount of biotin supplied by the liver should have been 30 to 50 gamma per dog per day, and should have brought about some response in hemoglobin. However, since there was a rapid increase in the body weight of these animals, and since the experiment was terminated prematurely by an epidemic of distemper, perhaps the hemoglobin did not increase rapidly enough to keep up with weight increase.

Lambooy and Nasset (2) maintain that some additional factors present in yeast are necessary for the growing dog. However, their animals were fed a high fat, low choline ration and received supplements of p-aminobenzoic acid and inositol. It is possible that such a diet either prevents the intestinal synthesis of some unknown factors or that it is deficient in some necessary factors itself. p-Aminobenzoic acid and inositol do not seem to be the cause of the increased demand for unknown factors since our dogs receiving these vitamins compared favorably with their controls. However, the high fat content of the ration used by Lambooy and Nasset might be responsible indirectly for the deficiency symptoms observed. This viewpoint is substantiated by the more recent work of Frost and Dann (8) in which they report none of the disturbances other than achromotrichia observed by Lambooy and Nasset when their dogs were fed a

diet containing only 3.5 per cent fat but high levels of choline. However, our dogs which were kept on a high fat ration for 6 months developed none of the deficiency symptoms described by Lambooy and Nasset (2) and Smith (3). There remains then the difference in levels of choline fed. These workers fed exceedingly low amounts of choline to their animals, and even though large amounts of protein were also fed it is possible that a choline deficiency existed. Fouts (9) found that under the conditions of a high fat diet, large amounts of choline (100 mgm./kgm./day) were required to prevent loss of body weight, anemia, dermal and peptic ulcers and death. More recently McKibbin (10) has shown that the choline requirement of the growing puppy is probably not more than 50 mgm. per kgm. of body weight per day. Both Fouts and Lambooy and Nasset cured the deficiencies in their animals by feeding yeast and liver. But it should be remembered that variable amounts of choline were furnished through these materials. Certainly the possibility of a choline deficiency must be considered since we find that our high fat, high choline ration will produce healthy dogs without yeast or liver supplementation.

SUMMARY

Under the experimental conditions of our laboratory dogs will grow well and maintain a good state of health without receiving any B vitamins other than thiamin, riboflavin, pyridoxine, pantothenic acid, nicotinic acid and choline. However, when a highly purified alcohol extracted casein ration is fed, hemoglobin will plateau at 11 to 14 grams per cent and severe achromotrichia often develops. Data are presented which indicate that biotin is necessary for the production of hemoglobin values greater than 14 grams per cent. Values of 16 to 16.5 grams per cent were obtained when dogs were supplemented with biotin and milk respectively for a 3 month period following a plateauing in hemoglobin.

None of the skin lesions and dermatitis observed by other groups of workers were found when our animals received a high fat ration. These differences are thought to be due to the level of choline feeding; our animals received levels of 50 mgm. of choline per kgm. of body weight per day whereas the other workers fed exceedingly low levels of choline.

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GONADOTROPIC ACTIVITY OF EQUINE GONADOTROPIN IN COMBINATION WITH ZINC

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It has been demonstrated that zinc and copper salts will augment the gonadotropic activity of anterior pituitary extracts in the rat (1). On the other hand, equine gonadotropin (pregnant mare serum) which has a marked gonadotropic activity in hypophysectomized animals is not augmented by either copper or zinc (2-4). In fact, Deanesly (5) observed a significant decrease in gonadotropic activity when the hormone was combined with zinc sulphate and has suggested that zinc may further slow the absorption of the hormone so that the total dose is not completely utilized. In the present investigation it was deemed of interest to determine whether the amount of zinc combined with equine gonadotropin was a determining factor in the response and also to determine whether the response to a total dose of hormone given in daily injections or as a single injection produced the same degree of stimulation. Since hypophysectomized rats respond as well to a single injection of equine gonadotropin as to the daily injections when administered subcutaneously (6) the influence of zinc on absorption rate could be studied.

MATERIALS AND METHODS. Immature rats of the Long-Evans strain were initially injected at 22 to 24 days of age. Each animal was injected subcutaneously once daily for 5 days or received a single injection subcutaneously. All rats were autopsied 120 hours after the initial injection.

Each animal injected with equine gonadotropin (Gonadin¹) received a total dose of 20 I.U. alone or in combination with 0.5 mgm., 2.5 mgm. or 7.5 mgm. of zinc. The solutions were prepared for injection by the addition of a small amount of $n/10$ sodium hydroxide to the hormone in solution. To verify the alkalinity, a drop of phenolphthalein was added at the same time as the designated amount of zinc sulphate solution. Then sufficient sodium hydroxide to permit maximum precipitation of the zinc as zinc hydroxide in the presence of the hormone was added and the pH adjusted to approximately 8.5. The preparations were shaken before withdrawal for injections to permit uniform administration of precipitate and solution.

At autopsy, the fresh weights of the ovaries and uteri were obtained in experiments involving the female and the weights of the testes, seminal vesicles (including the coagulating gland and any contained fluid) and ventral prostates were obtained in the male rat experiments.

RESULTS AND DISCUSSION. The combination of zinc and equine gonadotropin had less gonadotropic activity than equine gonadotropin alone when adminis-

¹ The equine gonadotropin (gonadin) was generously supplied by Mr. D. H. Wonder, Cutter Laboratories, Berkeley, California.

tered in equally divided doses once daily for 5 days (table 1). The loss of activity was not proportional to the amount of zinc added. The largest amount of zinc used (7.5 mgm.), however, exhibited the greatest effect in reducing the activity of equine gonadotropin but this amount could have been toxic. Fevold et al. (2) reported that 1 mgm. of zinc provides maximum augmentation of anterior pituitary follicle stimulating hormone (FSH). Evans et al. (3) found 1 mgm. of copper sulphate to be toxic as determined by the loss of body weight of their test rats. Although the 7.5 mgm. dose of zinc was well in excess of the stipulated maximal amount, normal body weight increases were observed, and no direct evidence for a toxic effect is apparent.

It was of interest to determine whether the hormone activity was associated with the precipitate or the solution. Mixtures were prepared in the manner described. After standing for several hours in an ice chest the precipitate was

TABLE 1
Influence of equine gonadotropin (PMS) and zinc on female rats

NO. OF RATS	BODY WEIGHT AVERAGE		TREATMENT (TOTAL DOSE)	AVE. ORGAN WEIGHT	
	Start	End		Ovary	Uterus
Gonadotropic activity of PMS and zinc					
	<i>gram</i>	<i>gram</i>		<i>mgm.</i>	<i>mgm.</i>
15	44.8	51.5	None	12.0	32.9
11	45.2	58.3	20 I.U. PMS	90.9	137.9
17	43.5	56.8	20 I.U. PMS and 0.5 mgm. Zn	53.8	131.7
15	40.1	51.5	20 I.U. PMS and 2.5 mgm. Zn	61.9	112.2
16	43.7	57.2	20 I.U. PMS and 7.5 mgm. Zn	39.9	121.1
Gonadotropic activity of filtrates of PMS and zinc mixtures					
7	36.1	50.7	Filtrate from PMS and 0.5 mgm. Zn	29.0	66.2
7	37.3	53.0	Filtrate from PMS and 2.5 mgm. Zn	13.3	30.7
5	33.6	46.4	Filtrate from PMS and 7.5 mgm. Zn	13.1	30.1

removed by filtration and the clear filtrate tested for gonadotropic activity. A small amount of the hormone was in solution when the mixture was prepared so that 0.5 mgm. of zinc and 20 I.U. of equine gonadotropin was to be the total dose. This is apparent since the filtrate increased ovarian and uterine weight more than 100 per cent (table 1). The other combinations show that the hormone was entirely associated with the precipitate of zinc hydroxide. Under these latter conditions, opportunity for augmentation of hormone activity should be maximal.

Equine gonadotropin differs from anterior pituitary extracts in that it is slowly absorbed, slowly destroyed in the body and there is little if any excretion of this gonadotropic material. The prolonged action of equine gonadotropin has been demonstrated in hypophysectomized rats in which the same total dose administered as a single subcutaneous injection was found to be just as effective

as five subcutaneous injections given once daily (6). Deanesly (5) suggested that zinc may further slow the absorption of the hormone so that the total dose may not be completely utilized. To test this hypothesis, the hormone and zinc combinations were granted a longer time to act by administering the mixtures as a single subcutaneous injection and killing the rats 120 hours later. A constant dose of 20 I.U. of hormone alone and in combination with zinc revealed

TABLE 2

Influence of a single subcutaneous injection of equine gonadotropin (PMS) and zinc on immature rats

NO. OF RATS	BODY WEIGHT AVERAGE		TREATMENT (TOTAL DOSE)	AVE. ORGAN WEIGHT	
	Start	End		Ovary	Uterus
	gram	gram		mgm.	mgm.
15	44.8	51.5	None	12.0	32.9
12	34.9	48.7	20 I.U. PMS	65.5	105.1
7	34.4	50.7	20 I.U. PMS and 0.5 mgm. Zn	60.3	99.0
9	36.8	49.8	20 I.U. PMS and 2.5 mgm. Zn	83.0	92.3
5	39.0	54.0	20 I.U. PMS and 7.5 mgm. Zn	52.8	107.2

TABLE 3

Influence of equine gonadotropin (PMS) and zinc on male rats

Influence of equine gonadotropin (1:125) and progesterone						
NO. OF RATS	BODY WEIGHT AVERAGE		TREATMENT (TOTAL DOSE)	AVE. ORGAN WEIGHT		
	Start	End		Testis	Sem. ves.	Vent. prost.
Normal controls						
16	gram 47	gram 54	None	mgm. 484	mgm. 8	mgm. 33
Animals injected daily for 5 days						
7	62	73	20 I.U. PMS	625	49	82
7	60	69	20 I.U. PMS and 2.5 mgm. Zn	548	42	65
Animals received a single injection						
9	58	66	20 I.U. PMS	526	32	72
9	57	64	20 I.U. PMS and 2.5 mgm. Zn	522	26	59

that under these circumstances a significant inhibition of gonadotropic activity was not produced by the zinc (table 2). In fact, the combination of 20 I.U. and 2.5 mgm. resulted in an augmentation which is on the border line of being significant. These experiments suggest that if the hormone has sufficient time to act then zinc does not cause an inhibition.

It is well known that an action of the luteinizing hormone of the anterior pituitary is to stimulate the interstitial cells of the testis and indirectly increase

seminal vesicle weight and that this response has provided an assay method for this pituitary fraction (7). It has also been shown that equine gonadotropin may give very little evidence of its luteinizing action in hypophysectomized female rats whereas the same dosage when tested in hypophysectomized male rats produces a marked seminal vesicle weight stimulation (8). Therefore, male rats were also investigated as to their response to 20 I.U. of hormone and 2.5 mgm. of zinc. This was the only combination used since in the female a definite inhibition of ovarian weight increase was observed with the daily injection method and a "possibly significant" augmentation occurred when administered as a single subcutaneous injection. No significant alteration of the gonadotropic action of equine gonadotropin on seminal vesicle weight was induced by zinc (table 3). Although an apparent suppression of ventral prostate stimulation was suggested, the variability and the known greater sensitivity of seminal vesicle weight (6) voids its significance.

A zinc salt was chosen since its ability to augment anterior pituitary extracts appeared to depend upon delaying absorption. Copper salts appear to influence physiological mechanisms and unlike zinc they will ovulate the rabbit and fail to augment FSH in hypophysectomized rats although augmentation is obtained in normal rats (2). Copper salts will also induce pseudopregnancy when administered intravenously to adult rats in estrum (9). It seems necessary to determine whether zinc salts have an action on the rat's pituitary in addition to an influence on rate of absorption in an effort to explain the sex difference in response to equine gonadotropin and zinc.

SUMMARY

A combination of zinc and equine gonadotropin has less ovarian stimulating activity than equine gonadotropin alone when administered in divided doses. The lowered stimulating action is not proportional to the amount of zinc sulphate added. However, when the mixture is administered as a single injection no inhibitory action of the zinc is observed.

The activity of equine gonadotropin is not significantly influenced by zinc when tested in male rats.

A mixture of 0.5 mgm. of zinc and 20 I.U. of equine gonadotropin has its activity associated with the precipitate and the solution whereas all hormone activity is associated with the precipitate when 2.5 mgm. of zinc is used.

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LIVER WATER AND ELECTROLYTES IN HEMORRHAGIC SHOCK¹

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The importance of changes in the liver functions during shock has been indicated in previous work. This is exhibited by a rise in amino acid concentration of plasma (1), a rise in blood lactate and pyruvate (1, 3), a fall in liver glycogen and a decreased ability to synthesize glycogen from glucose (2), a fall in oxygen consumption of liver slices (4) and a decrease in the ability of liver slices from shocked rats to deaminate amino acids and synthesize urea from (d-1) alanine and ammonium lactate in vitro (5). These findings have been interpreted as indicating that the liver is peculiarly subject to anoxia resulting from a diminished rate of circulation owing to its dependence on portal blood for a large part of its oxygen (6).

In connection with some of the above work, livers were analyzed for certain inorganic constituents to see what changes in liver composition take place during the development of these functional disturbances. Clarke and Cleg-horn (7) reported potassium and phosphorus analyses of the livers taken from dogs and rats subjected to experimental shock. On the basis of wet weight, the livers of the rats show a decrease in potassium while those of the dogs show an increase. Since fat analyses were not carried out, the data cannot be expressed per unit of fat-free solids and hence the interpretation must remain uncertain.

EXPERIMENTAL PROCEDURES. Before subjecting white rats to the experimental procedures, they were fasted for 24 hours. The livers were all taken from living animals but several were moribund and many would have died in a few hours owing to the effects of the experimental procedures. The controls were run parallel to group 2 and rats 1 and 2. The livers were analyzed by methods used in previous studies (8). All values are expressed per 100 grams of fat-free solids. The experimental manipulations will be listed in the description of the data presented in table 1.

RESULTS. *Group 1* represents the controls fasted 24 hours, subjected to nembutal anesthesia for the same length of time as group 2 and killed 24 hours later. They were allowed water ad libitum but no food after the anesthesia. Since the liver composition was not significantly different in unanesthetized controls fasted for 24 hours, simple fasted controls are not included in this table. Note that liver chloride is higher than liver sodium, a finding indicating some intracellular chloride (8, 9). The potassium is fairly constant but liver phosphorus shows a direct variation with liver fat that makes the values quite variable from group to group. In the experimental groups, there are quite

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large variations in liver phosphorus which cannot be interpreted because of the variability in fat content.

Group 2 represents livers from six rats that were sacrificed 24 hours after non-fatal hemorrhages equivalent to from 2.86 to 3 per cent of the body weight. The analyses do not vary significantly from the controls. The glycogen content of these livers, previously reported (1), also did not differ from the controls in group 1.

Rats 1 and 2 were bled 2.85 per cent of their body weight under nembutal anesthesia in the course of one hour. Rat 2 was sacrificed 90 minutes later

TABLE 1

EXPERIMENT NO.	NO.	CONCENTRATION PER 100 GRAMS FAT-FREE SOLIDS							Na + K H ₂ O
		H ₂ O	N	Cl	Na	K	P	Fat	
Controls and hemorrhage without changes in the liver									
Group 1.....	8	gm. 280 ±7	gm. 14.6 ±1.7	mM. 12.2 ±1.6	mM. 11.0 ±0.5	mM. 39.6 ±0.4	mM. 48.1 ±0.4	gm. 17	mM./kgm. 180 ±4
Group 2.....	6	289	14.9	12.6	10.8	40.5	50.0	20	177
Rat 2.....	1	275	13.4	10.8	12.0	38.5	45.4	9	184
Hemorrhage with changes in the liver									
Group 3.....	9	282 ±10	13.5 ±0.4	16.4 ±1.0	19.7 ±1.9	28.7 ±2.2	37.2 ±2.4	10	172 ±12
Group 4.....	3	291 ±9	14.3 ±0.9	13.9 ±3.9	14.3 ±2.6	31.0 ±0.8	34.9 ±0.8	10	153 ±16
Group 5.....	4	334 ±30	15.0 ±0.3	17.8 ±2.3	6.8 ±0.5	61.8 ±4.8	42.0 ±1.9	19	208 ±14
Rat 1.....	1	310	14.4	16.4	21.2	32.9	42.0	10	186
Anoxia with changes in the liver									
Group 6.....	4	293 ±13	15.3 ±0.6	14.9 ±2.4	17.1 ±1.7	35.3 ±2.9	41.8 ±1.4	18	179 ±3
Group 7.....	3	299 ±15	14.7 ±0.3	12.8 ±2.2	15.7 ±2.5	33.3 ±2.6	38.6 ±1.8	14	165 ±8

while apparently in good condition, as is apparent in the lack of change in the non-protein amino nitrogen of whole blood (18 mgm. per cent before hemorrhage and 15 mgm. per cent at time of death). Rat 1 was killed one hour after the end of bleeding when death from shock seemed imminent and showed a rise in non-protein amino nitrogen of whole blood from 15 to 25 mgm. per cent, a change characteristic of fatal shock (10). Note that in rat 2 there is no significant change in liver water and electrolyte while in rat 1 there is a significant rise in sodium, chloride and water and a significant fall in potassium. Attention is directed to the fact that the rise in liver sodium is such that sodium becomes higher than chloride but not sufficiently in excess to be contained in the same

volume of extracellular fluid at normal concentrations of serum chloride and sodium.

Group 3 represents 9 unanesthetized rats bled sufficiently over a period of an hour to produce death from shock. They were killed from 2 to 60 minutes after the bleeding was stopped. There is no distinct separation of the findings dependent on the time of survival after bleeding. This is revealed both by the individual liver analyses and the non-protein amino nitrogen of the plasma. The latter varied from 13 to 17 mgm. per 100 cc. which is the level associated with fatal shock. The findings in the liver are like those of the individual rat 1. This is true of each individual liver analysis as well as the statistic average shown in the table.

*Group 4*² was treated like group 3 except that after fatal shock had been produced by bleeding for one hour, transfusions equivalent to the amount of blood lost were given. The non-protein amino nitrogen of the plasma remained at about the same level (13, 17 and 20 mgm. per 100 cc.) during the hour allowed for therapeutic effects of the transfusion to become manifest. The liver analyses show the same low potassium as untreated shock but significant return of sodium and chloride toward normal has developed. While the values are still apparently high, the elevations of sodium and chloride are not of high degree of statistical significance when compared to the controls. The statistical difference between the treated and untreated rats subjected to shock is significant with respect to chloride and sodium.

Group 5 represents four rats bled small amounts at hourly intervals until moribund, two dying after fourteen hours and two after twenty hours of bleeding. At this time their levels of non-protein amino nitrogen of whole blood varied from 13 to 20 mgm. per 100 cc. The group differs from groups 3 and 4 in that time had been allowed so that considerable hemodilution had developed. In group 3 the hematocrits changed very little while in group 5 the hematocrits at the end of the experiment ranged from 11 to 15 per cent.

In the livers of group 5 the most striking finding is the low sodium associated with high chloride and potassium. The potassium is at least 50 per cent higher than that of the controls. This finding indicates accumulation of potassium chloride in the cells since the concentration of univalent base in total liver water ($\text{Na} + \text{K} \div \text{H}_2\text{O}$) is 204 instead of 180. The findings probably indicate changes coming terminally as liver functions have almost ceased.

Groups 6 and 7 show the effects on liver composition of total anoxia for 1 hour. The type of preparation was described in detail previously (6). Briefly the entire gastrointestinal tract and portal vein were removed surgically leaving the hepatic artery as the sole blood supply of the liver. Complete anoxia was produced by clamping the artery; the effects of restoration of the arterial circulation could be studied by releasing the clamp on the hepatic artery. In group 6, the hepatic artery was clamped for one hour and the liver removed at this time. Similar preparations showed a rise of non-protein amino nitrogen of

² We are indebted to Dr. George Sayers for making the livers of this group available to us.

blood of from 1 to 2 mgm. per 100 cc. In group 7 the circulation by way of the hepatic artery was restored for two hours after being cut off for one hour. Similar preparations show a slight recovery of non-protein amino nitrogen as a result of restoration of the arterial circulation of the liver.

The liver analyses of group 6 are essentially like those of shock in group 3 except that there is not quite as great decrease in potassium and somewhat less increase in liver sodium and chloride. The difference between groups 6 and 7 is not certainly significant. Thus while there is no certain evidence of recovery of electrolyte in the liver, similar preparations showed only slight accumulation of non-protein amino nitrogen in the plasma (6) and some recovery in the rate of oxygen consumption of liver slices (11).

DISCUSSION. It is impossible precisely to interpret the data with respect to distribution of liver water between extracellular and intracellular fluids in the absence of any certain method of estimating the extracellular water. The presence of more chloride than sodium excludes an exclusively extracellular position of chloride in normal livers. The rise in liver sodium suggests that more chloride may become extracellular in shock but still chloride is not sufficiently low to contain sodium in the same volume of extracellular fluid as will contain all chloride. This conclusion is based on the liver concentrations and assumed normal values for serum chloride and sodium (108 and 153 mM. per kilogram of water). Hence the volume of fluid indicated by the ratio of total liver sodium to the concentration of sodium in serum water is the largest possible volume of extracellular fluid. It is likely that this volume is a little too large since there is probably a small amount of intracellular sodium.

Calculating the volume of extracellular water by the ratio of liver sodium to the concentration of serum sodium, table 2 was prepared. Average normal concentrations for sodium (153) and chloride (108) were assumed. $N \times 6.25$ was used to calculate intracellular protein. Extracellular water times the concentration of chloride was used to calculate extracellular chloride. Non-extracellular chloride was assumed to be intracellular. Table 2 gives a reasonable picture of the state of body fluids. The chief uncertainty in the assumptions is the extracellular position of sodium. ♦

According to this interpretation as shown in table 2, the first event is loss of intracellular water and potassium accompanied by increase in extracellular water. This finding is illustrated early in shock in rat 1 and group 3 and to a less extent in anoxia in groups 6 and 7. There is definite evidence of recovery of intracellular water but not potassium in group 4 which received a transfusion after production of "irreversible" shock by bleeding. There is little certain evidence of recovery of composition after restoration of the hepatic circulation in group 7 as compared to 6 but this may, in part, be due to the fact that the changes are not as advanced in group 6 as in group 3.

It should be kept in mind that the above interpretation rests chiefly on the assumption of the extracellular position of sodium. If one assumed that sodium replaced potassium within the cells, the data would indicate that the distribution of water in the cells had remained relatively constant but large amounts of

chloride had entered intracellular water. The amount of intracellular chloride would be so large that it seems more likely that the sodium remained largely extracellular. Absolute proof of either interpretation is lacking and future work may show that sodium displaces potassium within the cells to a limited extent and only moderate increase in intracellular chloride develops.

In group 5 the changes in liver composition are strikingly different. Owing to great excess of chloride which develops while sodium is decreasing, chloride has certainly entered the intracellular water. The increase in liver potassium indicates that it is largely potassium chloride that enters the cells. The decrease in extracellular water indicated by the low liver sodium may be part of the process of mobilizing sodium to support plasma volume which is indicated by the decrease in proportion of red cells in blood. The accumulation of potassium chloride in the cells might be interpreted as due to a breakdown in the normal permeability of cellular membranes. Since it involves chloride but not sodium,

TABLE 2

EXPERIMENT	PER 100 GM. FAT-FREE SOLIDS *		CONCENTRATION PER KG. INTRACELL. WATER		
	Extracell. water	Intracell. water	K	Cl	Protein
	gm.	gm.	mM.	mM.	gm.
Group 1.....	72	208	190	21	438
Group 2.....	71	218	186	23	427
Rat 2.....	78	197	195	12	425
Group 3.....	129	153	188	16	552
Group 4.....	93	198	156	20	452
Group 5.....	44	290	213	45	323
Rat 1.....	138	172	184	8	524
Group 6.....	112	181	195	15	526
Group 7.....	103	196	170	9	469

it seems more likely that the changes are conditioned by some alterations in the properties of the cytoplasm that accompany loss in enzyme activities.

It is clear that the changes in the water and electrolyte composition of the liver are consistent in magnitude and concurrent in time with the alterations in tissue metabolism previously described. In group 3, the changes in composition of the liver were produced as rapidly as other evidences of "irreversible" shock developed and in group 2 changes in liver water and electrolyte were not demonstrated after hemorrhages which did not produce evidence of diminished enzyme activity. When evidences of partial recovery occurred in group 4, they are manifest both in measurements of enzyme activity (5) and electrolyte composition. Similar restoration is only suggested but not proven in group 7 as compared to group 6 although in similar preparations the oxygen consumption of liver slices (Q_{O_2}) was 1.6 cubic millimeters of oxygen per milligram of initial dry weight after one hour of anoxia and was 3.2 when 2 hours of arterial circula-

tion had led to partial recovery. (The normal $Q O_2$ is 5.3.) It is likely that recovery of enzyme activity precedes and determines the rate of recovery of normal water and electrolyte content. Evidence is already at hand that enzyme activity is dependent on the electrolyte milieu (12), but it is unlikely that the alterations which we have demonstrated explain the loss of enzyme activity. For this reason it would be futile if not dangerous to try to restore the electrolyte composition by administration of potassium. The present work merely demonstrates that the electrolyte composition is dependent on normal activity of the liver. Studies have so far indicated that effective restoration of liver function has only been promoted in shock by procedures which improve circulation and oxygen supply.

SUMMARY

The water and electrolyte composition of the livers of rats subjected to bleeding and anoxia are reported.

Bleeding sufficient to produce fatal shock or anoxia induced by occlusion of the hepatic circulation for one hour is accompanied by a loss of 10 to 25 per cent of the liver potassium and an appreciable increase in liver sodium and chloride. With prolonged repeated small hemorrhages, liver potassium becomes 50 per cent greater than normal and liver sodium decreases about 50 per cent. Increase in liver water accompanies this last type of change.

The changes in liver composition occur at the same time and under the same conditions as the enzyme disturbance accompanying shock and anoxia. The changes in liver electrolyte and water are apparently the result of loss of normal activity of the liver cells and the usual chemical composition of the liver is probably dependent on cell activity rather than structural properties of the membranes.

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THE PRODUCTION OF ARTERIAL HYPERTENSION BY CHRONIC RENAL ARTERY-NERVE STIMULATION

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Since reducing the blood flow to the kidneys by mechanical constriction of the renal arteries produces hypertension (2, 4, 6, 14), it seemed desirable to ascertain whether decreasing renal blood flow by stimulation of renal vasoconstrictor nerves might likewise cause an elevation in blood pressure. It is known (3, 7, 10, 15) that active renal vasoconstriction occurs in response to stimulation of the nerves to the kidney. However, under the conditions used in prior experiments the response has been of short duration, presumably because of rapid fatigue.

Cressman and Blalock (1939) attempted without success to produce hypertension in dogs by chronic stimulation of the splanchnic nerves with an alternating current of 60 cycles per second. They were able to obtain a transient increase in blood pressure in only one of three dogs stimulated. A clue to the reasons for their failure to obtain hypertension appears to be supplied by the work of Maltesos and Schneider (1938-39). These investigators reported that stimulation of autonomic constrictor nerve fibers with sinusoidal currents of ten cycles per second or less caused vasoconstriction without fatigue in contrast to the rapid fatigue which occurred when higher frequencies were used.

Therefore it seemed desirable to study the effects of various types of electrical stimulation of the renal nerves over short and long periods of time on renal blood flow and arterial blood pressure. Since both the artery wall and its associated nerve fibers are stimulated by the method described below, the effects will be referred to as those of renal artery-nerve stimulation.

METHODS. Medium sized mongrel dogs of either sex were used as the experimental animals. Shielded electrodes of no. 18 fine silver wire molded in lucite were slipped over both renal arteries and their accompanying nerves. An obturator molded of plastic closed the slot in the electrode block so that the artery and its associated nerves were enclosed within a smooth tube. The lead wires were silver wire cables made from 24 strands of no. 36 fine silver wire insulated with spectacle tubing. The resistance of the electrodes when in place was 800 to 2000 ohms.

In the acute experiments various types of current were used as stimuli. Alternating sine wave currents varying from one to three thousand cycles per second were tested. Condenser discharges at frequencies of one to seventy per second and interrupted direct currents of constant voltage were also tested. In the chronic experiments a 2 cycle per second sine wave alternating current developed by a special generator was used exclusively.

Renal blood flow was estimated in the acute experiments using a modified

form of the Baldes and Herrick (1, 8) type thermostromuhr. The stromuhr was molded of lucite using silver and constantan thermocouple elements. Silver wire cable was used in the leads.

For the acute experiments the dogs were anesthetized with ether during the operative procedure and then maintained with intravenous chloral hydrate. Through a midline abdominal incision either a bipolar electrode was put on the right renal artery, and all nerve fibers surrounding it, or a unipolar electrode was put on each renal artery with its accompanying nerves. The thermostromuhr was placed on the right renal vein to estimate renal blood flow. Carotid blood pressure was recorded on a kymograph with a mercury manometer. After a suitable control period the effect on blood flow and blood pressure of renal artery-nerve stimulation was observed and recorded.

For the prolonged renal artery-nerve stimulation dogs were prepared by fashioning a van Leersum (18) loop around the left carotid artery. Blood pressure was determined by the Riva Rocci (16) method using a cuff 5 cm. wide. Normal blood pressure levels were determined during a training and control period lasting from several weeks to thirteen months. A shielded unipolar silver electrode was then applied to each renal pedicle using aseptic technique. Through a lumbar incision the renal artery and all associated nerve fibers were carefully dissected free of fat. The shielded electrode was slipped over the artery and nerves and its obturator tied in place. Electrodes were applied to both renal arteries with associated nerves in the same operation. The leads were brought out through a stab wound in the back. A moulded and cushioned hardware cloth saddle was used to protect the leads. In the first experiment the dog was kept in a canvas harness which restricted him from moving about. In the later experiments the dogs were placed in a narrow cage allowing them to lie down or stand at will, but not to turn completely around. The daily period of stimulation in the chronic experiments was between 20 and 22 hours. Blood pressure measurements were made and feeding was done during the remaining period in most instances. In one dog, stimulation was maintained during the period of blood pressure measurement. No striking difference in blood pressure was noted with either procedure, although the amount of exercise preceding the blood pressure measurements, and the general psychic state of the animal could be maintained more constant from day to day by making the blood pressure measurements before the daily cessation of stimulation.

RESULTS. 1. *Acute stimulation.* In experiments on ten anesthetized dogs, shielded electrodes were placed around the renal artery and its associated nerves as indicated above. This area was then stimulated with various types of electrical currents, i.e., continuous and interrupted constant voltage direct current, condenser discharges, and various frequencies of sine wave alternating current continuously and at intervals. During the period of stimulation renal blood flow was measured using a thermostromuhr applied to the renal vein, while blood pressure was recorded directly from the carotid artery.

Renal artery-nerve stimulation with current from an inductorium caused an

immediate but transient marked vasoconstriction with a rapid return of the renal blood flow to normal while stimulation was continued. Direct current stimuli of various durations and at various intervals did not successfully maintain vasoconstriction. Higher frequencies of alternating current, 60 to 2700 c.p.s., were found to result in early disappearance of the response with the return of the renal blood flow toward the initial level (table 1). Attempts were made to prevent the decline in response when using higher frequency alternating currents by interrupting the current at intervals. Stimulation for $\frac{1}{10}$ to $\frac{1}{2}$ second per second, and also stimulation for periods of one second in five was tested. These rest periods only served to slow the rate of decline of the response at the sacrifice of vasoconstrictor action. In a series of experiments condenser discharges were also used as stimuli. It was found that rapidly decaying condenser discharges led to early fatigue. In one experiment on dog 5 (table 1) a slowly decaying condenser discharge was used at a rate of 21 stimuli per second. This stimulus caused a decrease of renal blood flow which was maintained for a period of 30 minutes. However, rapidly decaying condenser discharges at a rate of 21 per second quickly led to loss of response.

In experiments on four dogs, sine wave alternating currents of one to five cycles per second were used as stimuli for periods varying from 4 to 86 minutes (fig. 1 and table 1). In all cases there was a decrease of renal blood flow. When stimulation was continued ten minutes or more renal blood flow was always reduced for some time after stimulation was discontinued. The post-stimulus vasoconstriction was not marked after shorter periods of stimulation. In one of the longer experiments a 1.2 c.p.s. alternating current of 5 ma. (4 volts, 800 ohms) was employed for a period of 83 minutes (fig. 1). With the onset of stimulation blood flow rapidly decreased from 190 cc. per minute to approximately 70 cc. per minute and then more slowly fell to slightly less than 40 cc. per minute. After the stimulus was discontinued the blood flow returned very slowly toward normal so that it was not until forty minutes later that the blood flow was again 180 cc. per minute.

There was no marked change of blood pressure during stimulation with low frequency alternating currents. Usually the blood pressure rose 4 to 10 mm. of Hg immediately after stimulation was started and then within a minute or two the pressure fell again to the previous level. Occasionally the blood pressure appeared to remain a few millimeters of mercury above the initial level during the period of stimulation, but since there was some variation of the blood pressure with the state of anesthesia this rise does not appear to be significant. In no case was there any decrease of blood pressure in response to the stimulation.

In three experiments on very old dogs there was no change in the renal blood flow during renal artery-nerve stimulation with low-frequency alternating current. This observation may be fortuitous but is reported because no technical flaws were evident in the experiments in question. Failure to obtain renal vasoconstriction in young adult animals occurred in only one out of seven such dogs.

2. *Chronic stimulation.* The chronic experiments were divided into two general types as follows:

TABLE 1

Renal blood flow (thermostromuhr) during short duration renal artery-nerve stimulation of various types

DOG NO.	TYPE OF STIMULUS	DURATION OF STIMULUS	BLOOD FLOW		TOTAL DURATION OF DECREASED BLOOD FLOW
			Control	Minimum during stimulation	
		min.	cc./min.	cc./min.	
5	C.D. 5.5/sec. Rapid decay	1	175	190	0 min.
5	C.D. 10.5/sec. Rapid decay	1.5	190	140	<30 sec.
5	C.D. 21/sec. Rapid decay	1.5	165	140	<1 min.
5	C.D. 21/sec. Slow decay	30	165	90	30 min.
1	Harvard inductorium	1	130	29	<1 min.
1	2700 c.p.s. 14 v.	1	100	90	<1 min.
2	300 c.p.s. 2.5 v.	1	78	58	<1 min.
5	300 c.p.s. 4 v. Interrupted $\frac{1}{2}$ sec/sec.	3	170	140	<2 min.
2	175 c.p.s. 2.5 v.	2	90	56	<2 min.
2	175 c.p.s. 7 v.	2	77	32	<1 min.
5	175 c.p.s. 2 v. Interrupted $\frac{1}{2}$ sec/sec.	3	175	110	<30 sec.
5	60 c.p.s. 1 v.	2	220	130	<30 sec.
5	60 c.p.s. 2 v.	2.5	180	110	<1 min.
5	60 c.p.s. 2 v. Interrupted $\frac{1}{2}$ sec/sec.	8	155	96	<5 min.
3	5 c.p.s. 10 v.	10	130	15	>10 min.
3	5 c.p.s. 15 v.	15	195	16	24 min.
4	5 c.p.s. 6.5 v.	61	100	45	>61 min.
4	5 c.p.s. 7 v.	86	145	41	>86 min.
4	2 c.p.s. 5 v.	4	123	22	4 min.
4	2 c.p.s. 6 v.	8	123	10	8 min.
6	1.2 c.p.s. 4 v.	83	190	35	113 min.
7	1.2 c.p.s. 4 v.	59	240	160	67 min.

C.D. = Condenser discharge. c.p.s. = cycles per second (sinusoidal alternating current). v. = volts.

1. Relatively short intervals of stimulation to determine whether or not each period of stimulation would produce an increase in blood pressure (dog 1).
2. Long periods of stimulation to ascertain the length of time that the blood pressure would remain elevated with continued stimulation (dogs 2 and 3, fig. 2).

Dog 1: The blood pressure during the control period of 13 months varied from 110/80 to 165/115. In the period immediately before the electrodes were applied the blood pressure was 140/90.

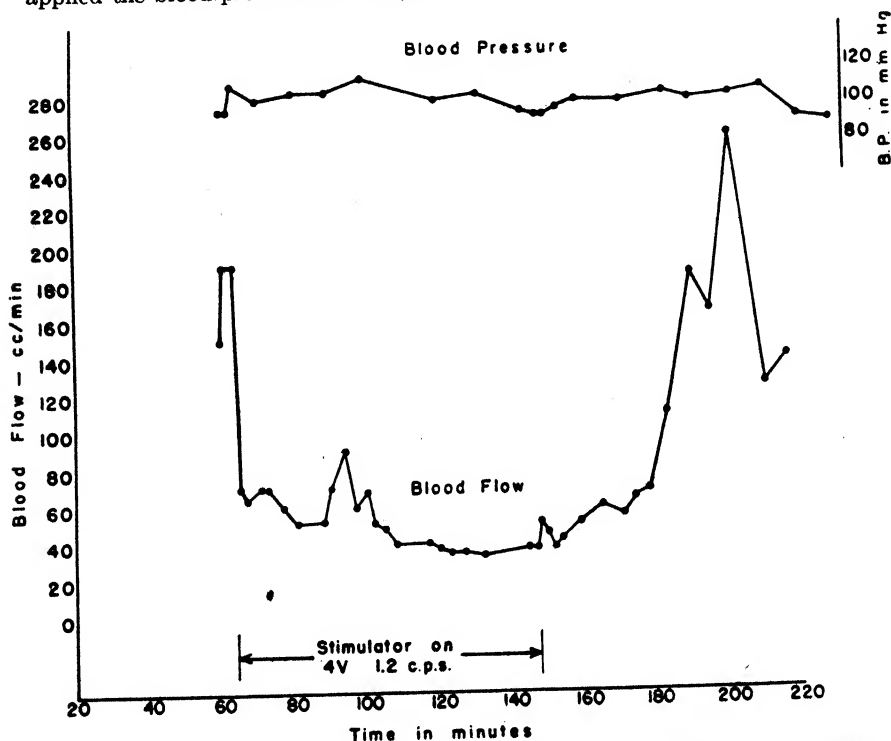


Fig. 1. The change in renal blood flow (thermostromuhr) and systemic blood pressure in relation to acute renal artery-nerve stimulation

Stimulation was started as soon as the animal had recovered from the anesthesia. Since there were no previous data available concerning an appropriate voltage for long time stimulation a voltage of 2 volts (peak) was used initially. The voltage was gradually increased to 4 volts over a period of about 4 days. Shortly after the stimulation was begun the blood pressure rose, fluctuating between 158/115 and 210/135. Stimulation was discontinued after four days of stimulation and the animal allowed to rest for 7 days. Blood pressure averaged about 165/115 during the seven day rest period. At the end of this period stimulation was again started at four volts (peak). On the second day of stimulation

the blood pressure had risen to 270/180 mm. Hg. Stimulation was discontinued for one day with a resultant fall in blood pressure to 170/130. Stimulation was then resumed at 4 volts. After 36 hours of stimulation the blood pressure had again increased to a value of 250/170. At this time the dog developed a staggering gait and would run into obstructions. About twenty-four hours later convulsions occurred. The blood creatinine at this time was found to be elevated from the control level of 1.0 mgm. per cent to 3.8 mgm. per cent. Cessation of stimulation was accompanied by rapid improvement in the condition of the animal. The electrode lead wires were accidentally broken on the 18th day after application, making further stimulation impossible. The blood pressure was followed for an additional 22 days with the electrode blocks on the renal pedicles. During this period the blood pressure slowly fell to 160/115. On

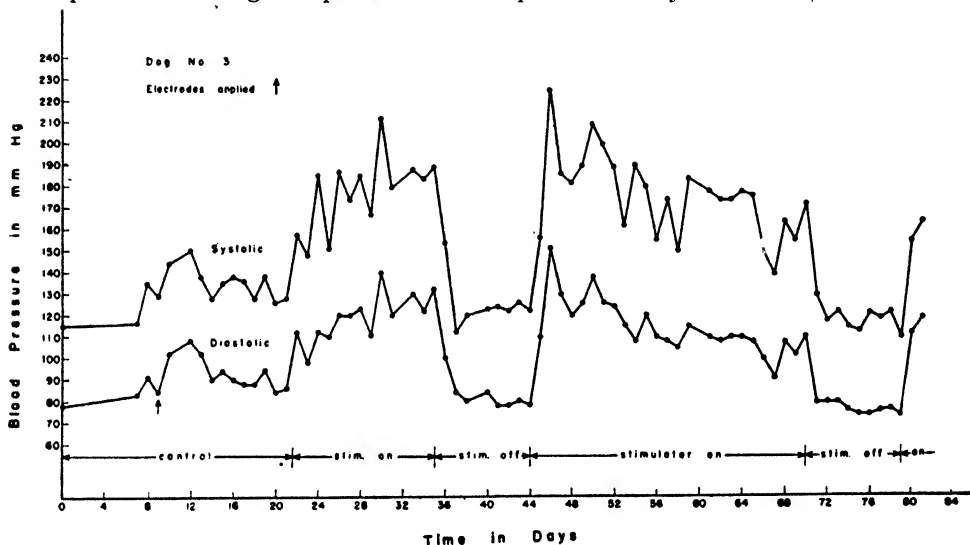


Fig. 2. Carotid arterial pressure in an unanesthetized dog in relation to chronic renal artery-nerve stimulation.

the 40th day after application the electrodes were removed using aseptic technique. The blood pressure was again measured for a period of about 40 days following removal of the electrodes. There was no marked change of blood pressure after the electrodes were removed.

Dog 2: During the control period of 21 days the blood pressure remained within the range 135/95 to 155/115. The mean blood creatinine level during this time was 1.3 mgm. per cent.

Application of the electrodes was followed by an increase in blood pressure to approximately 165/130 mm. Hg before electrical stimulation was begun. (The blood pressure rose in this experiment after applying the electrodes without stimulation.) Five days after the electrode application stimulation was started at 2.5 volts. The stimulating voltage was gradually increased to 4 volts over a

period of about 24 hours. During a period of 17 days of stimulation (22 hrs. per day) the blood pressure rose progressively to a plateau of approximately 210/165 mm. Hg. Cessation of stimulation for 8 hours was accompanied by a fall in blood pressure to 158/123. The stimulation was restarted and 12 hours later the blood pressure had increased to 198/160. The blood creatinine varied from 1.1 to 1.3 mgm. per cent during the period of stimulation. Stimulation was then discontinued for 7 days during which the blood pressure varied between 158/125 and 180/150. At the end of the 7 day rest period the stimulation was begun again. In 12 hours the blood pressure rose to 220/180 and remained at approximately this level for 9 days. At this time the blood pressure began to rise further to a maximum of 238/194 and the blood creatinine was 2.1 mgm. per cent. On the 47th day after application of the electrodes the animal died.

On histological examination of the kidneys by Dr. E. T. Bell, slight hemorrhages were observed throughout the cortex and medulla. However, both renal arteries were nearly occluded by thrombus formation in the region of the electrodes. Histological examination by Dr. Berry Campbell of the renal nerves both proximal and distal to the electrodes showed the nerves to be intact. It is inferred that this animal died from mechanical traumatic effects of the electrode block on the renal arteries. In subsequent experiments greater care was used in fitting the electrode blocks to the arteries.

Dog 3, figure 2: For a period of two weeks prior to making blood pressure studies this dog was placed daily for a half hour in the cradle used for blood pressure measurements. The dog was kept in the same cage and room throughout the entire experiment.

In an interval of nine days preceding application of the electrodes four blood pressure measurements were made which ranged from 115/78 to 135/91 mm. Hg. After application of the electrodes and before electrical stimulation was begun the blood pressure increased slowly for three days reaching a maximum of 150/108 mm. Hg, followed during the next two days by a gradual decline to 128/90 mm. Hg. Control blood pressures were recorded daily for eight additional days during which the blood pressure stabilized within a range of 126/84 to 138/94 mm. Hg.

Thirteen days after application of the electrodes stimulation was started at 1.5 volts at 2 c.p.s. Seven hours later the blood pressure was found to be 154/105 mm. Hg and 24 hours later 158/112 mm. Hg. Stimulation was gradually increased to 4 volts in the following two days. About 10 days after the beginning of stimulation (32nd day of the experiment) the blood pressure reached a plateau of approximately 185/125 mm. Hg. After 14 days of continuous stimulation (20-22 hrs. per day) stimulation was stopped. Twenty-four hours later the blood pressure had decreased to 154/100 mm. Hg and in 48 hours the blood pressure had fallen to 112/84 mm. Hg. After 6 days without stimulation the blood pressure stabilized at about 123/80 mm. Hg. Nine days after cessation of stimulation the stimulator was started again at 4 volts 2 c.p.s. Twenty-four hours later the blood pressure had increased to 156/110 mm. Hg and in 48 hours to 225/151 mm. Hg. Stimulation was continued for 27 days (20-22 hrs. per

day). For the first 14 days of this period the blood pressure slowly declined to a mean of approximately 165/110 mm. Hg and then became fairly constant. (Toward the end of the 27 day period the pressure remained at about 160/105 mm. Hg.) Cessation of stimulation was accompanied by an abrupt fall in blood pressure to 130/80 mm. Hg in 24 hours with the blood pressure stabilizing at about 120/85 mm. Hg after about 3 to 4 days without stimulation.

Frequency in relation to pain. It was observed in the acute experiments on anesthetized animals that the higher frequency sinusoidal currents, or faradic stimulation with an inductorium were more effective in stimulating pain fibers as indicated by a rise in blood pressure, labored respiration and dilatation of the pupil than the low frequency sinusoidal currents. At the same time these higher frequency currents were found to be relatively ineffective in causing vasoconstriction in the kidney. Observations on the relative effectiveness of high and low frequency sinusoidal currents for producing pain were also made on a conscious dog. When the renal artery-nerve of dog 2 was stimulated with a 60 c.p.s. alternating current of 2 volts for 15 seconds, there was maximal pupillary dilatation and the dog struggled violently. On the other hand a current of 2 cycles per second was maintained at 5 volts for 30 seconds with no response in pupillary dilatation, respiration, or voluntary movement. During the period of prolonged renal artery-nerve stimulation the animals showed discomfort only when the current was first turned on, as evidenced by mild struggling.

DISCUSSION. As was pointed out above it is well demonstrated that stimulation of the renal nerves can cause a temporary decrease of blood flow through the kidney. On the other hand denervation of the kidney has not uniformly been found to increase the renal blood flow (17). Milliken and Karr (13) reported that unilateral renal denervation in the dog caused an increase in blood flow and urine output which lasted for three months. In uninephrectomized dogs, Herrick, Essex and Baldes (9) reported an immediate increase in blood flow following denervation, but observed a gradual adjustment so that in time the renal blood flow returned to the initial level.

The acute experiments on anesthetized dogs reported here indicate that the renal blood flow can be drastically reduced by low frequency, low voltage stimulation of the renal arterial wall and its associated nerve fibers. Higher frequency stimulation leads to an early decline in the response, which may be due to fatigue. Condenser discharges with slow decay rates are superior to those with rapid rates of decay in obtaining prolonged vasoconstrictor effects. Sinusoidal alternating currents of 2 c.p.s. maintained vasoconstriction for the duration of the stimulation and a variable period thereafter, the post-stimulation effect being greater with the longer than with the shorter stimulation periods.

There was no significant blood pressure elevation in anesthetized dogs resulting from renal artery-nerve stimulation. This point is of interest in connection with the effects observed with chronic stimulation.

The chronic renal artery-nerve stimulation experiments have shown that it is possible to produce and maintain a profound arterial hypertension in dogs

by such means. In one animal a convulsive state associated with an elevated blood creatinine occurred coincident with prolonged stimulation and was relieved promptly upon cessation. In every case the arterial pressure fell toward normal after stimulation was discontinued. The rate of fall was not uniform. In some instances the return to pre-stimulation blood pressures was very prompt and not in others. It appears that a study of the conditions determining the rate of return to normal pressure after stimulation might be rewarding and such studies are under way.

Renal artery-nerve stimulation has been maintained for a maximum of 27 days, and blood pressure elevation has persisted during the same period. However, there is as yet no evidence that hypertension so produced has any important bearing on human essential hypertension. It would be necessary to show either that comparable nervous effects were operating continuously in the human disease, or that the renal artery-nerve stimulation would in time under proper circumstances produce an irreversible hypertension, in order to make the present results of great interest to the essential hypertension problem. However, there are many reasons for believing that nervous factors are of some importance in the genesis of human hypertension.

At present it is possible to state only that a severe, longstanding hypertension can be produced by appropriate renal artery-nerve stimulation, which persists at least for the duration of such stimulation. Observations on the mechanism of the effect upon the kidney and blood pressure will be presented in another paper (11). However, one point in this connection comes out of the studies reported here. The fact that blood pressure rises were not seen in anesthetized dogs stimulated for as long as 86 minutes during which time marked renal vasoconstriction occurred, appears to prove that the response observed with longer stimulation was not due to the simple hemodynamic effect of renal vascular constriction. Therefore it is inferred that the local vascular bed constriction does not mechanically raise the blood pressure in the chronic stimulation experiments. This deduction is further confirmed by the fact that maximal blood pressures are not ordinarily seen until 48 hours or more after stimulation is begun.

A neurogenic vasomotor effect on blood vessels elsewhere in the body resulting from chronic renal artery-nerve stimulation is not excluded but is unlikely since the effect may last 48 hours or more after cessation of stimulation.

SUMMARY AND CONCLUSIONS

1. Electrodes were placed around one or both renal arteries and their accompanying nerves in both acute and chronic experiments on dogs. Electrical stimulation with various types and frequencies of current were carried out in acute experiments. A sinusoidal alternating current of 2 c.p.s. was used in the chronic experiments. All types of stimulation produced temporary renal vasoconstriction.

2. Sinusoidal alternating currents of low frequency were more effective in causing maintained vasoconstriction than similar currents of higher frequency.

Condenser discharges with slow decay characteristics were superior to rapid decay condenser discharges.

3. In acute experiments renal blood flow was reduced 75 per cent for as long as two hours during renal artery-nerve stimulation without significant increase in arterial blood pressure.

4. Chronic renal artery-nerve stimulation (22 hrs. per day) resulted in an elevated blood pressure. With continuous stimulation the blood pressure remained elevated for as long as twenty-seven days. Cessation of stimulation was accompanied by a decrease in blood pressure.

5. Although renal artery-nerve stimulation in chronic experiments resulted in hypertension for the duration of the stimulation there is no evidence as yet that a more persistent hypertension can be produced by the means described.

6. The evidence indicates that the hypertension produced by chronic renal artery-nerve stimulation is not a simple hemodynamic consequence of kidney vascular bed constriction.

Acknowledgments. The authors are grateful to Mr. Wayne Adams for his indispensable and skillful technical assistance in these studies. They are also appreciative of the help of Drs. E. T. Bell and Berry Campbell in the interpretation of morphological aspects of the study and of Dr. Ernst Gellhorn for advice on stimulation techniques.

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FUNCTIONAL CHANGES IN NERVE AND MUSCLE AFTER PARTIAL DENERVATION¹

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This report deals with certain functional changes occurring in nerve and muscle after partial denervation and with attempts to enhance the reinnervation of denervated muscle fibers through the bifurcation of viable axones. The studies were carried out on the gastrocnemius muscles and tibial nerves of adult cats and albino rats. In these species the gastrocnemius muscles receive their motor innervation from several spinal nerves. This anatomical arrangement makes it possible to produce varying degrees of paralysis by section of one or more of the spinal nerves.

A large number of animals were employed inasmuch as it was known that considerable variability exists in different animals with regard to the segmental innervation of muscle. The studies represent completed experiments on a total of 116 rats and 15 cats. Partial denervation of the rat's gastrocnemius muscle was produced by removing a section of either L4, L5, or L4 and L5.² Three to four days were allowed to elapse for the severed axones to lose their excitability to electrical stimuli, after which the extent of initial paralysis was determined by measuring the isometric tension response of the gastrocnemius to tibial nerve stimulation. This value was compared with those from comparable tissues of the control contralateral unoperated limb. In addition, comparisons were made of the responses of the muscles to direct stimulation and of their wet weights. Comparable studies were made on other animals at 9, 14, 50 and 84 days after partial denervation. A number of experiments were carried out on both rats and cats in which one limb was partially denervated 3 days before and its contralateral a longer time before testing. The tension responses to direct and tibial nerve stimulations and the muscle weights on the two sides were then directly compared in the same animal. Control mock operations were carried out on a number of animals which included all steps in the procedure except for actual nerve section.

Attempts were made to facilitate the reinnervation of partially paralyzed gastrocnemii of rats by axone branching from viable axones. This was done by crushing the tibial nerve at the time L5 was sectioned. Studies were made upon the muscles and nerves of such animals at either 50 or 84 days after operation and the values compared with those from experiments in which only L5 was sectioned. The methods employed for stimulation of nerve and muscle and the measurement of isometric tension have been described in detail elsewhere (1).

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² The lumbar nerve is designated by the number of the respective vertebra below which it emerges.

The stimulus patterns were ones which caused maximal tension responses when applied to normal muscle and nerve.

RESULTS. The average values for the responses of the gastrocnemius muscles to tibial nerve and direct stimulation in relation to their contralateral controls at various times after partial denervation are summarized in figure 1 and table 1. The studies revealed a state of partial paralysis in the gastrocnemii of rats 3 days after spinal nerve section. Three days after the various spinal nerve lesions the algebraic sum of the residual tensions (nerve stimulation) which together constitute the total innervation of the gastrocnemius muscle results in a tension value approximating that of normally innervated muscle. However, when such a summation is made from the values recorded 14 days after operation, the result is considerably in excess of that found in normally innervated muscle. A considerable increase in the response to nerve stimulation was noted when the determinations were made at longer periods after partial denervation although meanwhile there was a loss in strength response to direct stimulation and in wet weight. This apparent partial recovery from the effects of spinal nerve section occurred rather abruptly between the third and ninth days and reached a plateau at about 14 days after operation (fig. 2). The responses to tibial nerve stimulation after the longer periods of partial denervation were always greater than those resulting from the activation of the contralateral tibial nerve at 3 days after operation. This increased response of partially paralyzed muscles to nerve stimulation can be shown to be an absolute one occurring during the time the muscles are undergoing further losses in weight and tension response to direct activation. Furthermore the loss in contractile strength evoked by direct stimulation of muscle and the rate of atrophy were definitely less than would be calculated from the values for the initial degrees of paralysis. Further recovery after 14 days in the capacity of nerve to activate muscle in the case of the rat was relatively gradual and slight (fig. 2). A partial recovery from the early effects of spinal nerve section was noted after L7 sections in the cat.

The results of the studies made on the gastrocnemius muscles and tibial nerves of animals subjected to combined L5 section and tibial nerve crushing offer no evidence for enhanced recovery. The data (fig. 3) show that at 50 and 84 days after operation the muscles were inferior in strength and weight to those which had been subjected singularly to either L5 section or crushing of the tibial nerve. At 84 days after crushing of the tibial nerve the rat's gastrocnemius muscles exhibit between 85 and 90 per cent of normal weight and strength (1). It appears that the advantages gained through reinnervation of denervated muscle fibers from branching axones were more than offset by incomplete reinnervation of the muscle as a whole. Our results are in agreement with the findings of Weiss and Campbell (2). These investigators have pointed out that crushing often fails to rupture the neurilemmal tubes and hence does not offer optimal conditions for effective reinnervation through axone branching. It appears entirely possible that under conditions more favorable for axone branching some improvement in the functional reinnervation of partially paralyzed muscle might take place.

DISCUSSION. Any satisfactory explanation for the early partial recovery of muscle from the effects of spinal nerve section suggests a consideration of several factors. Recovery of function by nerve and muscle following any degree of

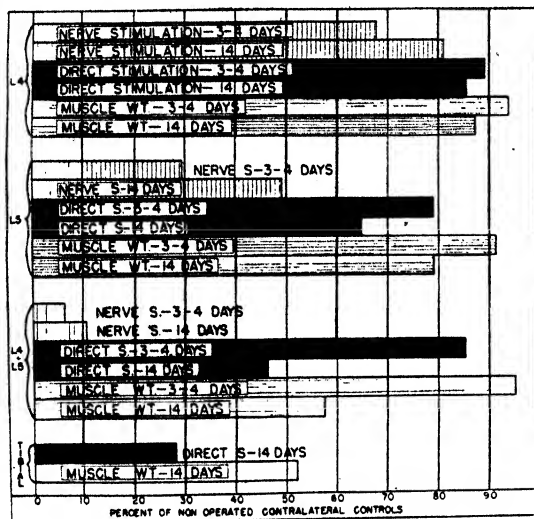


Fig. 1

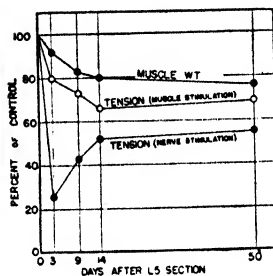


Fig. 2

Fig. 1. Changes in the weight and strength of rat's gastrocnemii following partial denervation. The average values include studies on 16 animals at 3 days and on 17 at 14 days after L4 section; 13 animals at 3 days and on 19 at 14 days after L5 section and on 6 animals at 3 days and on 6 animals at 14 days after combined L4 and L5 sections.

Fig. 2. The weight and strength of rat's gastrocnemii relative to unoperated contralateral controls at various times after section of fifth lumbar nerve. The average values include studies on 13 animals at 3 days, on 6 at 9 days, on 19 at 14 days and on 12 at 50 days after L5 section.

TABLE 1

A summary of average values for the gastrocnemii of fifteen cats subjected to bilateral section of seventh lumbar nerves

TIME AFTER SECTION	MUSCLE WEIGHT	TOTAL TENSION WHEN ACTIVATED THROUGH		TENSION PER GRAM MUSCLE WHEN ACTIVATED THROUGH	
		Nerve	Muscle	Nerve	Muscle
days	grams	grams	grams	grams	grams
3	12.683	3400	10,223	257	756
18	10.207	5809	8,023	557	730

peripheral nerve injury immediately raises the question as to whether the improvement could be related to a regeneration of the injured axones. In our experiments the greatest amount of recovery occurred in the two week period following partial denervation. In previous studies (1) on the rat the earliest

signs of functional reinnervation of the gastrocnemius muscle following denervation by crushing the tibial nerve did not make their appearance until about two weeks after operation. In those experiments the regenerating axones had only a relatively short distance to travel whereas after partial denervation the regener-

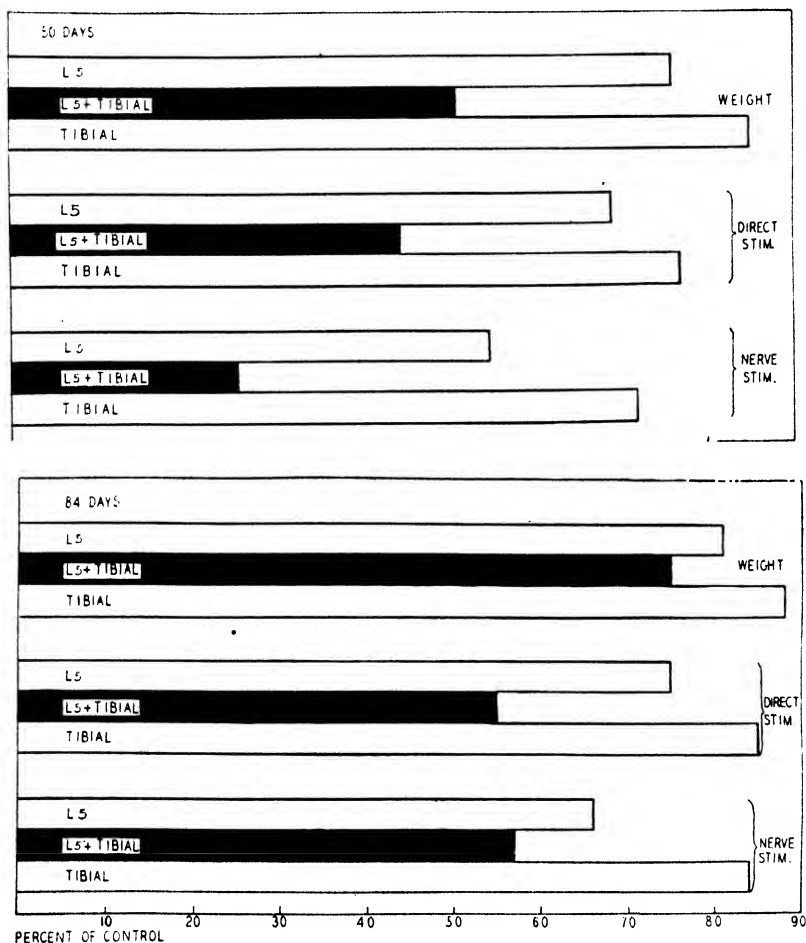


Fig. 3. Average values for the weight and strength of rat's gastrocnemii at 50 and 84 days after L5 section, combined L5 section and tibial nerve crush and tibial nerve crush only. The values include studies on a total of 18 animals in each group.

ating axones were required to grow a much greater distance and, also, traverse a gap and scar caused by the removal of a segment of the nerve. At sacrifice 14 days after operation no evidence of the bridging of the gap between the cut ends of the nerve could be seen. In order more completely to rule out the possibility that reinnervation might be responsible for the improvement in nerve function,

we made a careful inspection of the field of denervation in several cats and excised initial axone buds from the central processes of the severed nerves at the time of operation for the establishment of a second lesion in the contralateral limb. It would thus appear that the recovery in the capacity of the tibial nerves to elicit tension in their muscles cannot be attributed to a regeneration of the severed axones.

The question might be raised as to whether the recovery is an absolute one or only apparent because of changes in the controls. This error might be made if a unilateral lesion caused a retrograde loss of function in the control contralateral muscle and nerve. We have shown (3) that the section of the sciatic nerve to one gastrocnemius muscle is without effect upon the strength or weight of the unoperated contralateral muscle or the capacity of its tibial nerve to elicit tension in its muscle. Furthermore, the magnitude of the strength increases and their relationships when expressed on a body weight or gram muscle weight basis permits the conclusion to be drawn that the recovery is an absolute one and not an apparent one due to functional changes in the controls.

The negative findings in the mock operation experiments offer assurance that shock and trauma factors do not play any important rôles in these studies. This is interpreted to mean that the capacity of the nerve to elicit tension in its muscle at 3 days after partial denervation is not subnormal because of extraneous factors relative to the operation and that the subsequent functional improvement cannot be attributed to recovery from such states.

Consideration must be given to the possibility that the well known Vulpain-Heidenhain-Sherrington phenomenon enters into the explanation for the functional recovery noted in these experiments. It is well known that denervated muscle fibers show an increased sensitivity to acetylcholine soon after denervation and exhibit a contracture response. Could it be that the increased tension response of muscle some days after partial denervation represents an activation of the denervated muscle fibers by acetylcholine released from the activated viable nerve fibers? As to this possibility it can be said that the augmented response of the muscle to nerve activation occurred after a latency characteristic for normal muscle and nerve and that there were no signs of a contracture lag in any of the tension records.

One logical explanation might be that the increase in tension response of muscle to nerve stimulation was the result of hypertrophy of the normally innervated muscle fibers. The nature of our experimental data does not permit a satisfactory analysis of this possibility. However, such an explanation would require that the rates of hypertrophy exceed the rate of regeneration ordinarily found for muscles following reinnervation.

An attractive hypothesis can be offered for the increased command of nerve over muscle in that the phenomenon may represent a peripheral extension of the processes of viable axones to neighboring denervated muscle fibers or to an unmasking of normally functionless neuromuscular relationships. This concept would not be entirely foreign to peripheral nerve behavior after partial denervation. The studies of Weddell, Guttman and Gutmann (4) on the extension of

sensory nerves into denervated areas suggest that a comparable behavior of motor nerves might occur following partial denervation of skeletal muscle. Further exploration of the above mentioned hypotheses must await the evidence from careful anatomic studies.

It is generally recognized that a considerable degree of recovery from the initial paralysis often occurs in patients afflicted with poliomyelitis. Such recovery has been attributed to various conditions such as relief from a damaging local anemia and edema. It is interesting to note that the recovery found in our experiments occurred at comparable rat and human time equivalents after the onset of the initial partial paralysis. No information exists as to the conditions which might impede or enhance the rate and extent of this early spontaneous partial recovery from the effects of partial denervation. However, in evaluating the effects of any regimen of treatment directed toward the improvement of function following partial peripheral nerve paralysis from any cause, one should take into consideration the degree of functional recovery that will occur without treatment and not accept the degree of initial paralysis as an unchangeable baseline and attribute all improvement to the therapeutic regimen in question.

SUMMARY

A study has been made of the effects of partial denervation upon the gastrocnemius muscles of rats and cats. Partial denervation was accomplished by section of L4 or L5 or both in the rat and by section of L7 in the cat. At various times after spinal nerve section determinations were made of muscle weight and isometric tension responses to direct and to tibial nerve stimulations. The muscle and nerve of the contralateral limb were employed as controls. In some experiments these controls were unoperated; while in others, lesions comparable to those in the experimental members were made 3 days before testing.

The isometric tension responses to tibial nerve activation at 9, 14 or 50 days after operation were considerably greater than those found at 3 days after partial denervation. The improvement in the tension response of partially denervated muscle to nerve stimulation was not due to recovery from shock and trauma, regeneration of severed axones or weakness induced in the control muscle. Hypertrophy of the non-denervated muscle fibers and extension or unmasking of peripheral neuromuscular terminals are considered to be possible factors involved in the recovery of function following partial paralysis. The muscles of rats subjected to combined L5 section and tibial nerve crushing lesions were functionally inferior to those subjected to L5 section only when tested at 50 and 84 days after operation.

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BLOOD REGENERATION IN RATS DEFICIENT IN BIOTIN, THIAMIN OR RIBOFLAVIN

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In previous studies we have shown that a severe inadequacy of red blood cell regeneration is found in rats deficient in *L. casei* factor ("folic acid") and subjected to repeated hemorrhages (1); in pyridoxin-deficient rats, the rate of red blood cell regeneration is also impaired although not as markedly (2). There have been no blood regeneration studies in rats fed highly purified diets deficient in biotin or thiamin. The data in the case of riboflavin deficiency, as pointed out by Shukers and Day (3), are conflicting. In dogs with a partial deficiency of thiamin, Maas et al. (4) found no evidence of failure in red blood cell regeneration. However, riboflavin does appear to influence red blood cell regeneration in the dog as indicated by the work of György et al. (5) and Spector et al. (6), and in the monkey as reported by Waisman (7).

In the present studies, the red blood cell regeneration following hemorrhage in rats made deficient in biotin, thiamin or riboflavin was compared with blood regeneration in litter mates supplemented with the lacking vitamin. In addition, the red blood cell regeneration in rats fed an "adequate" purified diet (without added "folic acid") was compared with that of litter mates fed the same diet supplemented with "folic acid" concentrate from liver.

EXPERIMENTAL. Albino rats of Wistar or Osborne and Mendel strains were weaned at about 22 days and within one week thereafter were fed one of the experimental purified diets. Bleedings were made from the tail according to the technic of Tabor, Kabat and Rosenthal (8) as previously employed (1). Bleedings, equivalent to 2 per cent of the body weight, were made daily, every other day, or three times weekly as specified for each experiment. Hemoglobin determinations on tail blood were by the method of Evelyn (9); other procedures were as previously described (1).

Blood regeneration in rats fed purified diets with and without a supplement of "folic acid" concentrate. Ten male Osborne and Mendel rats were fed purified diet no. 988¹ and compared with 10 male litter mates of the same weight also fed diet no. 988 but given in addition an individual, daily, oral supplement of 0.029

¹ Diet no. 988 consisted of anhydrous dextrose 66.64 grams, Crisco 8.0 grams, casein (leached and alcohol-extracted) 18.0 grams, ferric citrate (iron 18.09 per cent) 1.16 grams, copper sulfate $\cdot 5\text{H}_2\text{O}$ 0.08 gram and salt mixture no. 550 (prepared according to the directions of Osborne and Mendel (10) except that the sodium fluoride was reduced to 1 per cent of their level and 0.313 gram of copper sulfate $\cdot 5\text{H}_2\text{O}$ is added) 4.0 grams. Into this diet were incorporated 1 mgm. of thiamine hydrochloride, 2 mgm. of riboflavin, 1 mgm. of pyri-

gram of liver concentrate containing 25 γ of *L. casei* factor ("folic acid")² (by assay with *Lactobacillus casei* E). Feeding was ad libitum and food intakes were measured. Bleedings were initiated the day after starting the experimental diet and repeated 3 times weekly for a total of 21 bleedings. The average values (fig. 1) in this small series of animals indicate no significant differences with respect to red blood cell regeneration, hemoglobin regeneration or body weight gain between the group of rats supplemented with "folic acid" concentrate and the group not supplemented. Average values for the supplemented group and non-supplemented group for total white blood cells per cu. mm. were 17,030 and 14,825 and for polymorphonuclear cells per cu. mm. were 2,175 and 1,705 respectively. Anemia or white blood cell dyscrasias³ were not noted in any rats in either group. The average food intake for the entire experimental period was approximately equal in both groups.

Blood regeneration in biotin deficiency. Rats were fed an egg white-containing diet no. 999⁴ similar to that used by Emerson and Keresztesy (12). Signs of severe biotin deficiency were developed in approximately 90 per cent of the rats in from 5 to 12 weeks. In all deficient rats marked cheilosis, dermatitis, alopecia, humped posture, abnormal gait and weight loss or poor weight gain were noted.

In *experiment 1*, bleedings were started when a rat was judged to be severely deficient according to the above criteria. In a group of 16 Wistar rats, bleedings were repeated every other day until interrupted by the death of one animal (in 10 cases after 7 to 24 bleedings) or by inability to bleed the rat due to the inadequate length of the tail (in 6 cases after 22 to 32 bleedings). No deaths occurred during the actual hemorrhage procedure. The data summarized in table 1 indicate that hematocrit values were maintained near a level of 40 vol. per cent, thus comparing favorably with the red blood cell regeneration of rats

doxine hydrochloride, 4 mgm. of calcium pantothenate, 2 mgm. of niacin, 200 mgm. of choline chloride, 0.001 mgm. of biotin, 0.4 mgm. of 2-methyl-1, 4-naphthoquinone, 2 grams of corn oil containing 16,000 units of vitamin A and 1,600 units of vitamin D (Natola), and 0.12 gram of ethyl laurate containing 12 mgm. of α -tocopherol.

² The liver concentrate was furnished through the courtesy of Dr. E. L. R. Stokstad of Lederle Laboratories.

³ The development of granulocytopenia in a small percentage of rats fed purified diets almost identical to diet no. 988 and the correction of this dyscrasia by crystalline *L. casei* factor has been reported (11). Anemia and granulocytopenia were noted in a rat (in another study) fed diet no. 988 and not subjected to hemorrhage. After a steady fall, the blood values were as follows: hematocrit 19 vol. per cent, hemoglobin 6.1 grams per cent, red blood cell count 3,290,000 per cu. mm., total white blood cell count 2,400 per cu. mm. and polymorphonuclear cell count 0. Crystalline *L. casei* factor (100 γ) was administered orally for each of 4 days. The blood values on the 5th day after the start of treatment were as follows: hematocrit 37 vol. per cent, hemoglobin 11.4 grams, red blood cell count 4,670,000, total white blood cell count 45,100 and polymorphonuclear cell count 15,000. Also, a sharp gain in body weight occurred following treatment.

⁴ Diet no. 999 consisted of egg white (Armour) 15.0 grams, casein (Labco) 15.0 grams, Crisco 10.0 grams, salt mixture no. 550 4.0 grams, cod liver oil 2.0 grams, dried pork liver 2.0 grams, ferric citrate 1.16 grams, copper sulfate $\cdot 5 \text{ H}_2\text{O}$ 0.08 gram and sucrose 50.76 grams. Into this diet were incorporated thiamin, riboflavin, pyridoxin, calcium pantothenate, niacin, choline, biotin and vitamin K as in diet no. 988.¹ Once weekly each rat received a supplement of 3 mgm. of α -tocopherol in 0.03 cc. of ethyl laurate.

fed an adequate purified diet (fig. 1) (1). In addition, no difference in the rate of red blood cell regeneration was found between those rats which died during the series of hemorrhages and those that survived them. Even during the terminal state of the animal, regeneration appears to have been maintained at or

TABLE 1
Lack of impairment of blood regeneration following repeated hemorrhage in biotin-deficient rats

GROUP	RAT NO.	BODY WEIGHT		NO. OF DAYS ON EXPERIMENTAL DIET BEFORE 1ST HEMORRHAGE	NO. OF HEMORRHAGES	HEMATOCRIT VALUES (VOL. %)											Recovery period Values at 2 and 4 days after last hemorrhage			
		At initial hemorrhage	At final hemorrhage			Hemorrhage period(Bleedings every other day) Values prior to hemorrhages														
						1st	4th	6th	8th	10th	12th	20th	Last before death							
														2	4					
EXPERIMENT 1																				
Biotin-deficient rats which died during the series of hemorrhages	1	99	85	44	7	54	44	43	†					42						
	2	79	63	44	14	53	41	46	45	45	44	†		42						
	3	91	66	44	16	53	41	39	41	36	38	†		35						
	4	96	85	44	12	53	35	41	37	41	50	†		50						
	5	76	67	44	12	53	40	37	41	40	46	†		46						
	6	130	116	44	15	52	33	47	36	41	38	†		29						
	7	87	63	44	21	51	34	45	39	46	44	34		33						
	8	115	94	50	24	54	37	39	43	49	42	30		36						
	9	126	126	80	15	44	32	35	36	38	37	†		40						
	10	150	130	80	10	53	32	36	39	30	†			30						
Average		105	80			52	37	43	40	41	42		38							
Biotin-deficient rats which survived the series of hemorrhages	11	129	114	44	32	54	41	40	45	48	48	39		38			41			
	12	96	114	44	24	48	39	40	34	36	40	39		44			44			
	13	97	99	44	24	50	40	45	41	42	46	40		40			46			
	14	103	124	80	27	52	38	45	43	37	33	38		38			35			
	15	126	124	80	22	51	38	41	38	43	39	35		34			39			
	16	170	209	80	27	55	33	42	40	38	40	39		38			39			
Average		120	131			52	38	42	40	41	41	38		39			41			
EXPERIMENT 2																				
Biotin-deficient rats	No. of rats.....					8	8	6	6	6	6	4		4			4			
	Average	84	87	38		51	43	43	44	41	42	39		43			36			
	Range					46-54	37-58	39-47	41-47	35-48	35-49	35-45		36-54			35-37			
Biotin-supplemented rats	No. of rats.....					8	8	7	7	7	7	7		1			7			
	Average	81	134	38		49	40	45	44	40	41	40					34			
	Range					43-53	32-44	40-44	39-48	37-44	37-49	36-43		32			30-38			

† Dead.

near a normal rate. It should be noted that a significant loss of weight occurred in the rats which died during the series of hemorrhages but not in the rats that survived them.

The details of experiment 2 are essentially the same as those of experiment 1,

except that here a comparison was made between biotin-deficient rats and pair-fed litter mates supplemented with biotin. When the experimental diet had been fed (*ad libitum*) for 31 days (5 pairs) or 50 days (3 pairs) and a severe biotin deficiency had been developed, the rats (Wistar) were divided into 2 groups of approximately equal weight, sex and litter distribution. One group received 20 γ of crystalline biotin subcutaneously twice weekly; the other group received no supplement. Rats given biotin were limited in their food intake to that of biotin-deficient litter mates or of deficient rats of comparable weight when litter mates had died. Amelioration of the signs of deficiency was noted within a few days after the administration of biotin and no stigmata remained after 3 to 4 weeks. Hemorrhages were initiated in both groups the day following the first biotin injection in the supplemented group and repeated three times weekly for a total of 24 times unless interrupted by death of the animal. Four rats in the biotin-deficient group died after 5 to 15 hemorrhages. The data in table 1 indicate that no significant difference exists between the rate of red blood cell regeneration in biotin-deficient rats as compared with biotin-supplemented rats. The average mean corpuscular hemoglobin concentrations calculated from hemoglobin determinations made at intervals during the series of hemorrhages were 30.4 and 31.6 per cent for the biotin-deficient rats and biotin-supplemented rats respectively.

Blood regeneration in chronic thiamin deficiency. Thirty-six Wistar rats divided into 4 groups of equal weight, sex and litter distribution were fed a thiamin-deficient diet no. 947² and supplemented as follows:

Group A, C— 2 γ thiamin up to the 30th day and 1 γ thereafter

B —100 γ thiamin (pair-fed with group A)

D —100 γ thiamin (fed *ad libitum*)

The supplements of thiamine hydrochloride were administered orally by pipette each day. After 20 days on the experimental diet, groups A, B and D were subjected to hemorrhage for 3 consecutive days. Beginning six days after the third hemorrhage, another series of 3 daily hemorrhages was carried out. Hematocrit values are listed in table 2; the values of chief interest are those obtained during the recovery period following each series of hemorrhages. The hematocrit values of the thiamin-deficient rats (group A) were somewhat lower during this period of red blood cell regeneration than the values for the pair-fed, 100 γ thiamin-supplemented litter mates (group B). Although these differences are statistically significant when litter-mate comparisons are made by Student's method (13) (P values <0.05), yet they are very slight and are only suggestive of an underlying impairment in red blood cell regeneration. It is noteworthy that regeneration to normal or near normal values occurred in both groups A and

² Diet no. 947 consisted of anhydrous dextrose 68.76 grams and Crisco, casein (Labco), ferric citrate, copper sulfate and salt mixture no. 550 in the same quantities as in diet no. 988. Into this diet were incorporated riboflavin, pyridoxin, calcium pantothenate, niacin, choline, biotin and vitamin K as in diet no. 988.¹ Twice weekly each rat received a supplement of 0.25 cc. of corn oil containing 2,000 units of vitamin A and 200 units of vitamin D (Natola) and once weekly 3 mgm. of α -tocopherol in 0.03 cc. of ethyl laurate.

TABLE 2

Effect of chronic thiamin deficiency on erythropoiesis following hemorrhage

RAT ^a NO.	HEMATOCRIT VALUES (VOL. %)												SUR- VIVAL IN DAYS
	Hemorrhage period (Bleeding daily) Values prior to each hemorrhage			Recovery period Values at 2, 4 and 6 days after 3rd hemorrhage			Hemorrhage period (Bleeding daily) Values prior to each hemorrhage			Recovery period Values at 2, 4 and 6 days after last hemorrhage			
	1st	2nd	3rd	2	4	6	4th	5th	6th	2	4	6	
DAYS ON EXPERI- MENTAL DIET	20	21	22	24	26	28	36	37	38	40	42	44	
Group A—Thiamin: 2 γ from 0 to 30th day, then 1 γ from 31st day to end of experiment													
1A	49	35	26	28	41	43	51	37	23	23	33	41	45
2A	46	33	23	26	42	43	52	35	23	23	37	37	65
3A	47	33	24	31	38	44	50	31	23	24	31	31	45
4A	51	34	19	23	34	38	49	33	21	23	39	43	75
5A	51	29	22	21	35	40	46	26	17	15	†		41
6A	45	37	24	23	37	42	49	32	16	19	34	43	83+
7A	47	32	21	22	34	41	45	31	21	18	23	30	52
8A	43	38	24	25	41	48	52	40	15	23	36	40	45
9A	43	34	29	21	42	42	50	30	21	25	39	44	75
Average.....	47	34	24	24	38	42	49	33	20	21	34	39	

Group B—Thiamin: 100 γ —Pair-fed with Group A

1B	46	31	26	33	42	41	55	39	27	28	43	49
2B	44	32	27	35	45	48	51	35	23	26	38	42
3B	49	35	26	28	40	45	50	28	23	26	38	42
4B	42	34	23	26	36	40	51	34	26	30	40	45
5B	42	33	26	31	40	47	50	36	27	30		
6B	46	32	21	25	38	45	50	35	21	26	38	42
7B	48	31	24	24	40	45	45	34	28	24	31	37
8B	41	31	17	22	38	44	49	38	24	22	35	37
9B	51	35	25	30	43	47	55	35	25	29	41	46
Average.....	46	33	24	28	40	45	50	35	25	27	38	43

Group D—Thiamin: 100 γ —Fed ad libitum

Average.....	44	31	24	29	40	42	45	28	20	25	39	
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* Numeral indicates number of litter; letter indicates group.

Rats in group C, supplemented as rats in group A but not bled, had an average hematocrit of 50.7 vol. per cent after 45 days on experimental diet.

The average body weights after 20 days on the experimental diet were 78, 78, 76 and 91 grams for groups A, B, C and D respectively and after 44 days the weights were 61, 64, 63 and 143 grams for groups A, B, C and D respectively.

† Dead.

B after both series of hemorrhages despite a pronounced weight loss due to inanition during this period.

Rats in the thiamin-deficient group C which were not bled had an average

hematocrit of 50.7 vol. per cent (range: 47-53) after 45 days on the experimental diet. However, much lower values (29, 33, 34, 38 and 44 vol. per cent) were noted in the 5 rats which were alive after 63 to 68 days on the experimental diet.

Blood regeneration in acute thiamin deficiency. In three separate but similar experiments, Wistar, and Osborne and Mendel rats in 3 groups equal with respect to weight, sex and litter distribution were fed thiamin-deficient diet no. 947.⁵ The groups were divided and given the following supplements:

Group A, C—no thiamin supplement

Group B—100 γ of thiamine hydrochloride each day orally by pipette (paired with group A). After 11 to 16 days on the experimental diet, bleedings were initiated in groups A and B and repeated every other day for the total number of times indicated in table 3. Rats in group C served as non-bled, thiamin-deficient controls. (In expt. 3, rats in groups A and B were given 20 γ of crystalline *L. casei* factor⁶ orally for each of 5 days starting on the 16th experimental day in order to make for a more complete vitamin supplement.) The data of all 3 experiments are summarized in table 3. While hematocrit values under 20 vol. per cent were reached in 11 of 17 thiamin-deficient rats (A groups), none of the pair-fed, thiamin-supplemented rats (B groups) had such low values. Nine thiamin-deficient rats which remained alive for 6 days after the last bleeding showed no evidence of increase in hematocrit values during this period. Among the thiamin-deficient rats which were not bled (C groups) the lowest hematocrit values for each rat ranged from 30 to 56 vol. per cent (average: 44.4). Only 2 values under 40 vol. per cent (30 and 36) were noted. The average survival time after starting the experimental diet was 29 days in this group.

Blood regeneration in riboflavin deficiency. Wistar rats were fed a riboflavin-deficient diet no. 963⁷ or no. 990⁸ in 3 separate experiments. The period of preparation on these diets prior to the initiation of bleedings was varied in the different experiments in order to produce varying degrees of riboflavin deficiency.

In a preliminary study (*experiment 1*) 28 rats divided into 4 groups of equal weight, sex and litter distribution were fed diet no. 963⁷ and supplemented as follows:

Group A, C—No riboflavin

B —200 γ riboflavin (pair-fed with group A)

D —200 γ riboflavin (fed ad libitum)

Supplements of riboflavin were administered orally by pipette each day. After 22 days on the experimental diet groups A, B and D were subjected to a series of 3 daily hemorrhages. Twelve days following the third hemorrhage, the rats

⁵ Crystalline *L. casei* factor (14) was furnished through the courtesy of Dr. E. L. R. Stokstad of Lederle Laboratories.

⁷ Diet no. 963 differed from diet no. 947⁵ in that riboflavin was omitted, thiamine hydrochloride (1 mgm. per 100 grams of diet) was included and sucrose replaced anhydrous dextrose. The diets and fat-soluble vitamin supplements were otherwise identical.

⁸ Diet no. 990 was the same as diet no. 963⁷ except that anhydrous dextrose and leached, alcohol-extracted casein were used instead of sucrose and Labco casein respectively.

TABLE 3
Effect of acute thiamin deficiency on erythropoiesis

GROUP	RAT* NO.	HEMATOCRITS (VOL. %)								Recovery period Values at 2 and 6 days after last hemorrhage	
		Hemorrhage period (Bleedings every other day) Values prior to hemorrhages									
		1st	2nd	4th	6th	8th	10th	last†	2	6	
Experiment 1											
Days on experiment..		11	13	17	21	25	29				
A—no thiamin	1A	44	29	27	32	13	†	13			
	2A	37	27	30	32	29	27	20	17	18	
	3A	39	27	33	37	32	24	17	12	9	
	4A	49	34	28	32	†		16			
B—100 γ thiamin	1B	43	31	36	37	39		39	44	50	
	2B	30	34	32	30	37	34	38	34	39	
	3B	39	28	36	39	38	40	35	36	41	
	4B	45	34	30	38			41	30	36	
Experiment 2											
Days on experiment..		16	18	22							
A—no thiamin	1A	50	31	26				26		19	
	2A	48	37	21				21		†	
	3A	50	30	14				14		15	
	4A	43	28	16				16		†	
	5A	42	26	17				17		11	
	6A	40	30	26				26		28	
B—100 γ thiamin	1B	49	24	†				20			
	2B	45	33	†				33			
	3B	46	26	30				30		33	
	4B	41	31	21				21		†	
	5B	45	31	30				30		42	
	6B	46	33	33				33		32	
Experiment 3											
Days on experiment..		16	18	22	26						
A—no thiamin + “folic acid”	1A	49	41	31	26			26	18	16	
	2A	47	40	37	37			37	37	33	
	3A	53	43	31	31			31	17	†	
	4A	52	43	30	17			17	10	†	
	5A	47	42	35	37			37	29	25	
	6A	50	40	26	26			26	†		
	7A	41	40	37	38			38	†		

TABLE 3—*Concluded*

GROUP	RAT* NO.	HEMATOCRITS (VOL. %)								Recovery period Values at 2 and 6 days after last hemorrhage	
		Hemorrhage period (Bleedings every other day) Values prior to hemorrhages									
		1st	2nd	4th	6th	8th	10th	last†	2	6	
Experiment 3— <i>Concluded</i>											
Days on experiment..		16	18	22	26						
B—100 γ thiamin + “folic acid”	1B	54	40	35	35			35	†		
	2B	50	40	35	35			35	†		
	3B	47	43	†				24			
	4B	54	40	†				39			
	5B	49	40	28	36			36	†		
	6B	51	42	34	50			50	†		
	7B	47	44	35	31			31	27		
Average of A groups								23			
Average of B groups								34			

* Numeral indicates number of litter; letter indicates group.

† Dead.

‡ Hematocrit values prior to last hemorrhage before death of a rat or death of its litter mate or after 12, 4 and 6 hemorrhages in experiments 1, 2 and 3 respectively.

were subjected to a second series of 3 daily hemorrhages. The average hematocrits at the initial hemorrhage were equal in all 3 groups. In the riboflavin-supplemented groups B (restricted food intake) and D (ad libitum food intake), the average hematocrit values during the hemorrhage and recovery periods were approximately equal. The values were significantly lower, however, in the riboflavin-deficient rats (group A). Thus the average hematocrit values at 2, 4 and 6 days after the third hemorrhage were 22, 30 and 37 vol. per cent, respectively, for group A, as compared with 27, 36, and 43 vol. per cent respectively, for group B, and 26, 40 and 44 vol. per cent respectively for group D. At 2, 4 and 6 days after the sixth hemorrhage, the average hematocrit values were 22, 28 and 34 vol. per cent, respectively, for group A as compared with 30, 40 and 43 vol. per cent, respectively, for group B and 30, 42 and 41 vol. per cent respectively for group D. In the riboflavin-deficient rats which were not bled (group C), hematocrit values under 35 vol. per cent (24, 32 and 33) were noted in 3 rats.

In *experiment 2* (table 4), Wistar rats were fed a riboflavin-deficient diet no. 9637 for 31 days. At this time they were divided into 3 groups (A, B and C) of 15, equal in weight, sex and litter distribution. As in experiment 1, rats in groups A and C were continued on the diet without supplementation and each rat in group B received 200 γ of riboflavin orally by pipette each day. For a more complete vitamin supplement, 10 γ of *L. casei* factor⁶ were administered orally to rats in all groups for 10 days starting on the 33rd experimental day.

TABLE 4
Effect of riboflavin deficiency on erythropoiesis

RAT* NO.	HEMATOCRITS (VOL. %)												
	Hemorrhage period† (Bleeding daily) Values prior to each hemorrhage			Recovery period Values at 2 and 4 days after 3rd hemorrhage		Hemorrhage period‡ (Bleeding every other day) Values prior to hemorrhages						Recovery period Values 2 and 4 days after last hemorrhage	
	1st	2nd	3rd	2	4	4th	6th	8th	10th	12th	last	2	4
	33	34	35	37	39	41	45	49	53	57			
Days on experiment	33	34	35	37	39	41	45	49	53	57			
Group A—No riboflavin													
1A	49	38	27	28	37	42	33	34			27	25	31
2A	49	38	26	25	37	41	38	27	20	†	15		
3A	42	25	25	29	37	41	29	26	28	27	25	27	38
4A	47	38	33	30	37	40	26	26	25	27	26	21	36
5A	48	28	22	26	33	†					33		
6A	47	34	27	26	34	40	32	†			22		
7A	56	36	25	26	38	47	34	30	24	23	32	28	38
8A	46	38	26	23	35	40	30	27	25	17	19	26	†
9A	49	39	28	28	38	43	35	31	29	24	20	†	
10A	45	33	25	25	34	39	25	21	†		21		
11A	48	37	30	30	43	48	38	31	25	†	25		
12A	46	33	23	26	39	41	33	27	26		26	24	29
13A	50	33	25	23	31	42	23	23	23	22	30	30	22
14A	52	40	25	28	41	45	39	31	32	33	41	40	47
15A	43	30	22	28	35	42	29	24	22	19	19	†	
Average.....	48	35	26	27	37	42	32	28	25	24	25	25	34
Group B—200 γ riboflavin													
1B	47	38	32	38	48	49	43	38	†		38		
2B	52	38	29	29	41	50	44	36	38		39	36	30
3B	45	37	32	37	42	48	41	39	31	36	37	36	46
4B	46	36	30	31	46	49	38	35	36	38	33	29	40
5B	52	34	23	13	15	†					15		
6B	44	39	36	36	50	44	34				40	43	45
7B	46	36	33	32	46	55	36	38	40	38	31	30	42
8B	54	40	30	32	46	49	42	42	40	42	41	45	46
9B	48	34	28	33	47	47	42	44	40	42	33	40	43
10B	48	33	27	30	42	42	39	37			37	41	40
11B	51	44	35	39	48	52	34	36	37		37	42	49
12B	50	36	29	32	46	50	42	41	39	†	39		
13B	48	35	29	31	43	52	40	38	40	38	44	39	46
14B	53	38	31	27	40	48	37	38	38	42	43	40	46
15B	50	32	24	26	41	46	36	40	32	37	37	33	35
Average.....	49	37	30	31	43	49	39	39	37	39	36	38	42

* Numeral indicates number of litter; letter indicates group.

† Dead.

‡ Average total hemoglobin removed per rat in group A was 0.30 gram and in group B 0.32 gram.

§ Average total hemoglobin removed per rat in group A was 0.56 gram and in group B 0.92 gram.

Rats in group B were pair-fed with rats in group A. Two days after setting up the three groups, a series of 3 daily hemorrhages was initiated in group A and B. Four days after the third hemorrhage, another bleeding was carried out and repeated every other day until the death of the rat or its litter mate, or until the completion of 14 hemorrhages. The data (table 4) indicate that the values in the riboflavin-supplemented rats (group B) were maintained at near normal hematocrit levels (37-39 vol. per cent) while the values in riboflavin-deficient rats (group A) declined to hematocrit values around 25 vol. per cent. This difference appears even more significant when it is noted (table 4) that during the period of alternate-day bleedings, an average total of only 0.56 gram hemoglobin per rat was removed from the riboflavin-deficient rats (group A) as compared with 0.92 gram from the riboflavin-supplemented rats (group B). Among the non-bled, riboflavin-deficient rats (group C), 3 were observed to have hematocrit values under 35 vol. per cent (16, 26 and 26).

Experiment 3 (fig. 2) was carried out for the purpose of making hemoglobin determinations and examinations for bartonellosis (15) in addition to repeating the findings of experiments 1 and 2. Wistar rats were fed a riboflavin-deficient diet no. 990.⁸ After 38 days on this diet they were divided into 2 groups (A and B) of 7, equal in weight, sex and litter distribution. As in experiment 2, rats in group A were maintained on the diet without supplementation while each rat in group B received 200 γ of riboflavin daily and was pair-fed with its litter mate in group A. A daily, oral supplement of 25 γ of crystalline *L. casei* factor⁶ was administered to each rat in both groups for 6 days starting on the 38th day. Two days after setting up these 2 groups, rats were subjected to the initial hemorrhage; bleedings were repeated 3 times weekly until the death of the rat or its litter mate or until the completion of 9 hemorrhages. Smears were made from the tail blood of each rat prior to every bleeding, stained by the giemsa method and carefully examined for bartonella-like inclusions in the red blood cells. Hemoglobin determinations by the Evelyn method were made from tail blood prior to each bleeding along with hematocrit determinations. The hematocrit values (fig. 2) again reveal the impairment in the rate of red blood cell regeneration found in riboflavin-deficient rats in the preceding experiments. In addition, the lower values for mean corpuscular hemoglobin concentration in the riboflavin-deficient rats indicate an even greater inadequacy in the rate of hemoglobin regeneration than is indicated by the hematocrit values. Examinations of smears for bartonella bodies failed to reveal characteristic organisms in any of the rats.

Mention has been made of the occurrence of anemia in some riboflavin-deficient rats which were not subjected to hemorrhage. In addition, granulocytopenia and leukopenia have been noted in many riboflavin-deficient rats both among those that were bled and those that were not, but not among *L. casei* factor ("folic acid")-supplemented rats. The nature of these dyscrasias is being reported separately (16).

DISCUSSION. In the present studies it was found that riboflavin-deficient rats manifested a moderate impairment in red blood cell and hemoglobin regeneration

following hemorrhage. The impairment was slight in thiamin-deficient rats, while in biotin deficiency no significant effect on red blood cell or hemoglobin regeneration was noted. Previous studies have indicated that erythropoietic inadequacies are found in rats with "folic acid," pyridoxin or pantothenic acid deficiencies (17) (1) (2) (18).

Although it has been demonstrated that blood regeneration is impaired when certain vitamins are lacking in the diet, the mechanism whereby a particular vitamin exerts its influence on blood regeneration in a rat deficient in this vita-

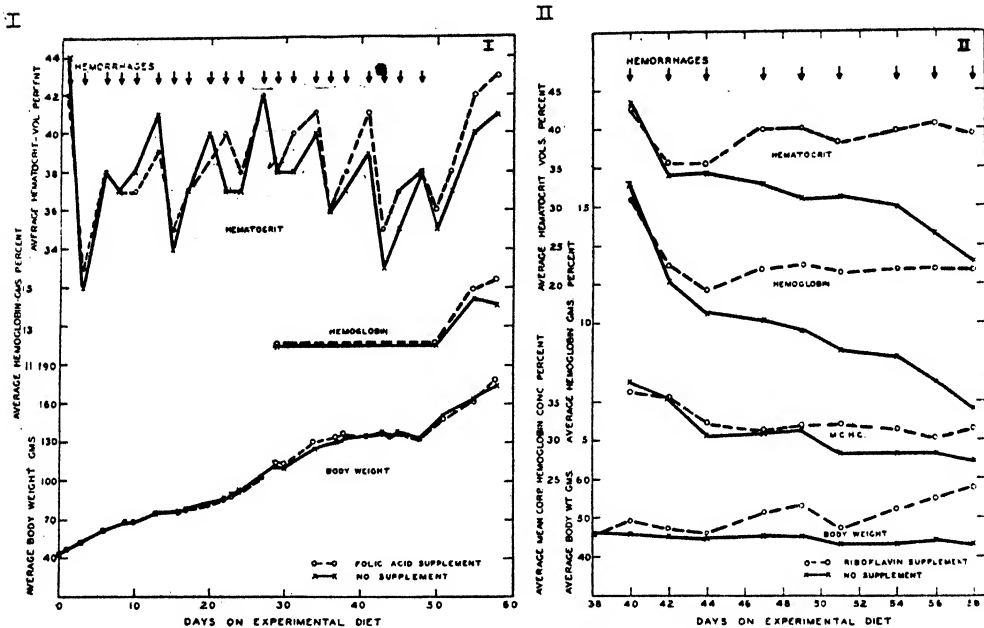


Fig. 1. Blood regeneration in rats fed purified diets with and without a supplement of "folic acid" concentrate.

Fig. 2. Blood regeneration in riboflavin-deficient rats compared with pair-fed, riboflavin-supplemented littermates.

Average total hemoglobin removed per rat in the riboflavin-deficient group was 0.82 gram and in the riboflavin-supplemented group 1.11 gram.

min remains obscure. It is clear, however, that the vitamins differ greatly in their relative importance in this function. Contrasting biotin with "folic acid" may serve to illustrate this point. In approximately 50 per cent of the biotin-deficient rats studied in these experiments, there was a progressive decline in weight terminated by death. Yet, despite this failure to maintain body weight, these rats were able to replace their red blood cell volume a number of times at a rate equal to that of biotin-supplemented controls or rats fed an "adequate" purified diet. However, "folic-acid" deficient rats with little or no weight loss

were almost totally unable to regenerate red blood cells or hemoglobin (1). A similar, though less striking, contrast may be made between thiamin and riboflavin. In the face of severe weight loss in thiamin-deficient rats, only a slight impairment in red blood cell regeneration was found, while in riboflavin-deficient rats, the inadequacy in red blood cell and hemoglobin regeneration was moderate despite the absence of any notable weight loss.

The data in these studies of vitamin-deficient rats are based entirely upon peripheral blood concentrations. Measurements to ascertain alterations in total circulating red blood cell or plasma volume have not been made. Such alterations, if present, might modify conclusions concerning red blood cell regeneration in which slight or no impairment has been found.

It is of interest that supplementation with "folic acid" of a group of 10 rats fed an "adequate", purified diet (without added "folic acid") did not produce a significant increase in the rate of red blood cell or hemoglobin regeneration as compared with non-supplemented rats. In work previously reported (11) it was shown that only a small percentage of rats fed such an "adequate", purified diet develop signs of "folic acid" deficiency (as compared with the uniformly high incidence of the deficiency when sulfonamides are included in the diet). Thus it seems probable that when such an "adequate" purified diet is fed, a large number of rats must be studied in order to find individuals that develop a deficiency of "folic acid" and as a result manifest an impairment in red blood cell regeneration.

*SUMMARY

Peripheral blood studies of the regeneration of red blood cells and hemoglobin following repeated hemorrhages have been made in rats fed vitamin-deficient diets.

Biotin deficiency resulted in no significant effect on red blood cell or hemoglobin regeneration.

Thiamin deficiency produced a slight impairment in the rate of red blood cell regeneration.

Riboflavin deficiency resulted in a moderate impairment in the rate of red blood cell and hemoglobin regeneration. Anemia was noted in some riboflavin-deficient rats not subjected to hemorrhage.

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THE EFFECT OF CALCIUM CONCENTRATION ON PROTHROMBIN TIME

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It has been previously reported by us (Jaques and Dunlop, 1945) that the prothrombin time of the blood of dogs treated with dicumarol is very sensitive to changes in the concentration of calcium. The optimal concentration was found to be 0.025 to 0.050 M, while the prothrombin time was doubled at concentrations of 0.10 and 0.01 M. At the peak of the dicumarol effect, no clotting could be obtained with these latter concentrations. Dilution of normal plasma failed to duplicate this effect of calcium concentration, the same prothrombin being obtained with concentrations of calcium from 0.001 M to 0.1 M. Likewise, dilution of the thromboplastin, while it lengthens the prothrombin time, does not cause any increased variation in its value with calcium concentration.

In an attempt to establish the reason for this effect of calcium on the dicumarol plasma, further studies have been made of the effect of calcium concentration on the clotting of normal plasma.

METHODS. Oxalated plasma from normal dogs was used in all experiments. The blood sample was obtained by drawing it into a syringe containing 1/10 volume of 0.10 M sodium oxalate. Alumina plasma was prepared from this by adding to it one-tenth volume of Alumina Cy, incubating for 10 minutes at 37°C. and centrifuging. The prothrombin time was determined by the method of Quick (1938), using 0.1 cc. plasma + 0.1 cc. of thromboplastin + 0.1 cc. of calcium chloride solution. Prothrombin times were done in duplicate. Determinations were made with calcium chloride from 0.001 to 0.25 M. The thromboplastin was prepared from acetone-extracted rabbit brain by heating 0.5 gram of dried brain in 10 cc. of saline at 56°C. for 15 minutes and taking the supernatant suspension.

The two-stage determination of prothrombin was conducted as modified by Herbert (1940) and Irish, Hamilton, Haist and Jaques (1945). For fibrinogen a lyophilized preparation of human fibrinogen, kindly supplied by Prof. E. J. Cohn, was used.

Effect of calcium concentration on the prothrombin time of alumina plasma. Quick (1935) has shown that prothrombin can be removed from plasma by adsorption with alumina gel. Provided contact with gel is not too prolonged, prothrombin appears to be the only clotting factor removed in significant amounts. The prothrombin time with varying concentrations of calcium was therefore determined for plasma after treatment with alumina gel. The results obtained on two plasma samples are shown in figure 1. As reported previously, variations in the calcium concentration between 0.01 and 0.1 M had little effect on the prothrombin time of normal plasma. However, following treatment with

alumina, the concentration of calcium had a marked influence on the prothrombin time. The prothrombin time for 0.025 M calcium was increased from 10 seconds to 41 seconds, representing a decrease in prothrombin to about 5 per cent of the initial concentration. The optimal calcium concentration was found to be 0.05 M and the prothrombin time increased rapidly with concentrations above and below this value. No clot could be obtained with 0.2 and 0.01 M calcium. With the second sample a rather high normal prothrombin time was obtained. After treating this with alumina, no clot could be obtained with 0.025 M calcium, indicating almost complete removal of the prothrombin according to the Quick prothrombin time. Prothrombin times of 100 seconds with 0.05 M calcium, and of 2 minutes 40 seconds with 0.10 M, were obtained. It is evident, therefore, that the effect of calcium observed with dicumarol plasma can also be found in plasma treated with alumina. Further, as with the dicumarol plasma, the

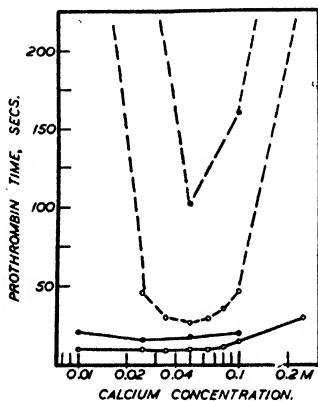


Fig. 1. Effect of calcium concentration on prothrombin time. — normal plasma. --- same plasma after treatment with alumina. ○—○ plasma 1, ●—● plasma 2.

effect is more marked, the lower the concentration of prothrombin present, as judged by the Quick prothrombin time (i.e., at 0.025 M calcium).

Effect of calcium concentration in the two-stage method. Actual measurements of prothrombin concentration in plasma can be secured by the use of the two-stage procedure. This was therefore conducted with varying concentrations of calcium. The results are shown in table 1 and are reported as the actual clotting times obtained on adding a sample of the activated plasma to the fibrinogen-buffer solution. It can be seen that the maximum amount of thrombin was obtained with 0.05 M calcium. A significant decrease in the yield was obtained on using 0.1 and 0.01 M. The values represent the amount of thrombin obtained during the period of maximal activity. This does not represent the total prothrombin present since, as shown by Herbert, some destruction of thrombin occurs in these tests.

Table 1 shows the effect of calcium concentration on the maximum amount of thrombin formed. In order to study the effect of calcium concentration on the

rate of activation, activation mixtures were made up containing 0.05 cc. of each of thromboplastin, calcium, buffer, and diluted oxalated normal plasma. After incubation for various times at 25°C., 0.1 cc. of the fibrinogen solution was added and the clotting times at 37°C. determined. The results are reported in table 2 for two samples of normal plasma a 1:50 dilution being used in one case, a 1:100

TABLE 1
Effect of calcium concentration on the two-stage technique

M Ca CONCENTRATION	THROMBIN CLOTTING TIMES DILUTION OF PLASMA		
	1:100	1:200	1:400
	<i>sec.</i>	<i>sec.</i>	<i>sec.</i>
0.1	34	50	74
0.05	28	40	62
0.025	32	44	67
0.01	39	66	108
0.005	38	58	

TABLE 2
Effect of calcium concentration on rate of activation of prothrombin

M Ca CONCENTRATION	THROMBIN CLOTTING TIMES		
	30 sec.	1 min.	4 min.
	<i>sec.</i>	<i>sec.</i>	<i>sec.</i>
Plasma A, 1:50			
0.5	∞	∞	∞
0.1	166	110	59
0.05	128	62	42
0.025	102	48	42
0.01	107	57	41
0.005	107	63	43
0.0025	177	67	48
0.001	∞	∞	210
Plasma B, 1:100			
0.5	∞	∞	∞
0.1	∞	∞	275
0.05	310	225	106
0.025	240	148	80
0.01	430	250	104
0.005	∞	330	130
0.0025	∞	∞	285

in the other. It can be seen that the rate of activation of the prothrombin, as judged by the amount of thrombin formed after 30 seconds and 1 minute incubation, is affected by the concentration of calcium present. The maximum rate of activation was obtained with 0.025 M calcium. This corresponds to 0.008 M calcium in the activation mixture. The optimum found previously for

the prothrombin time of oxalated plasma corresponded to 0.008 to 0.013 M, while that for dicumarol blood without added oxalate was 0.008 M. If we assume that at the high dilution of the plasma used in this test there is complete dissociation of the oxalate present, then the optimum concentration reported corresponds to that previously found by the determination of prothrombin times.

As seen in the experiments on alumina plasma, the effect observed was greater with the more dilute prothrombin concentration. It should be pointed out that in diluting the plasma, not only is the prothrombin diluted but also the thrombin inhibitors present in plasma. Their action is largely though not completely removed at these dilutions. As shown by Mertz, Seegers and Smith (1940) in plasma the amount of thrombin formed represents a balance between the rate of activation of prothrombin and the rate of destruction of the thrombin by the inhibitors. Hence, the effect of calcium on the rate of activation shown in table 2 will be aided in undiluted plasma by the destruction of thrombin proceeding simultaneously.

In conclusion, it is evident that the concentration of calcium is an important factor in the rate of activation of prothrombin. This effect of calcium is seen on determining the prothrombin time of alumina plasma and dicumarol plasma, where the prothrombin concentration has been reduced to a low level, the effect increasing with the decrease in prothrombin. However, in normal plasma there is a great excess of prothrombin, and only about 1 per cent is activated in the 10 seconds to 15 seconds of a normal prothrombin time. It seems reasonable to conclude, therefore, that the effect of calcium concentration on the activation rate of the prothrombin in normal plasma is compensated by the great excess of prothrombin present, so that it does not materially affect the prothrombin time.

SUMMARY

The effect of calcium concentration on the clotting of alumina plasma and on the activation of prothrombin in normal plasma has been studied. The prothrombin time of alumina plasma shows the same sensitivity to calcium concentration previously found with dicumarol plasma. The rate of activation of prothrombin is sensitive to changes in calcium concentration and hence the latter affects the prothrombin time at low concentrations of prothrombin.

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THE EFFECT OF THIOURACIL ON THE RESPIRATION OF BONE MARROW AND LEUCOCYTES *IN VITRO*^{1, 2}

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It is now widely recognized (1-4) that the clinical use of thiouracil in the treatment of hyperthyroidism carries with it the hazard that serious leucopenia or even fatal agranulocytosis may occasionally occur. It consequently seemed desirable to determine whether thiouracil has any demonstrable effect on the respiratory metabolism of the bone marrow and polymorphonuclear leucocytes of an experimental animal. For if the drug were found to have a depressant effect on the respiration of myeloid tissue, it would be possible to make *in vitro* tests of agents proposed to protect the marrow from this manifestation of an undesirable action. In particular, we hoped to determine whether pyridoxine, which has recently been recommended (5, 14) for the treatment of agranulocytosis, exhibits any antagonism, demonstrable *in vitro*, to the action of thiouracil.

METHODS. Rabbit femoral and tibial bone marrow was employed, the techniques for handling this tissue for measurement of respiration in the Warburg apparatus having been previously worked out (6, 7, 8). The marrow samples were blotted on filter paper and the wet weights determined with a torsion balance before placing them in the vessels. Autogenous partially-neutralized (8) serum was used throughout as medium, and the respiration of each set of marrow samples was determined with and without added thiouracil in final concentration of 100 mgm. per cent. This concentration is much higher than that in the serum of patients being treated with the drug, but it has been shown (9) that in persons receiving thiouracil in therapeutic amounts, the drug is highly concentrated in the bone marrow, reaching levels comparable with the above. Most of the determinations were made in triplicate, the others in duplicate. The results reported below are based on the rates of respiration found during the last hour of 3-hour experiments, the depressant action of the drug being slightly progressive during this period.

Marrows of various cellular composition were obtained by injecting the animals from 3 to 19 days earlier, either with acetylphenylhydrazine intraperitoneally to produce erythroid metaplasia or croton oil intrapleurally to produce myeloid metaplasia (10). Previous experience has indicated that neither of these drugs, in the amounts used, affects the respiratory metabolism of the marrow cells. In each experiment, the proportion of myeloid and erythroid cells present was determined by making differential cell counts on marrow smears stained with Wright-Giemsa.

¹ A preliminary report of these studies has appeared in *Science* **102**: 175, 1945.

² This work was aided by a grant from the John and Mary R. Markle Foundation.

For comparison with the marrow experiments, suspensions of leucocytes were obtained by injecting the rabbits intraperitoneally with several hundred cubic centimeters of Ringer solution in the evening and withdrawing the exudate the following morning. It has been shown (11), and our own observations confirm, that at least 95 per cent of the cells so obtained are polymorphonuclear leuco-

TABLE 1
Effect of thiouracil on bone marrow respiration in vitro

EXP. NO.	TREATMENT DRUG†; DAYS	PER CENT MYELOID CELLS	CONTROL QO ₂ *	PER CENT CHANGE IN QO ₂ IN MEDIUM CONTAIN- ING THIO- URACIL	EXP. NO.	TREATMENT DRUG†; DAYS	PER CENT MYELOID CELLS	CONTROL QO ₂ *	PER CENT CHANGE IN QO ₂ IN MEDIUM CONTAIN- ING THIO- URACIL
Predominantly erythroid marrows									
16	P.H. 3	34	0.69	-13	32	P.H. 5	15	0.89	-8
28	P.H. 3	32	0.99	-7	33	P.H. 10	27	0.89	+1
29	P.H. 5	19	0.76	+4	35	P.H. 2	39	0.52	+5
30	P.H. 4	17	0.58	0	41	P.H. 5	26	0.76	+5
31	P.H. 4	16	0.93	-5	42	C.O. 6	37	0.52	+3
Intermediate group									
2	C.O. 3	48	0.45	-11	18	C.O. 19	56	0.50	+3
10	C.O. 6	42	0.58	+3	20	C.O. 3	45	0.50	-9
11	P.H. 3	51	0.88	-5	23	C.O. 5	46	0.69	-7
13	C.O. 6	59	0.34	+2	34	C.O. 3	43	0.52	-3
14	C.O. 3	50	0.51	+2	38	C.O. 3	58	0.34	-11
15	C.O. 4	54	0.59	+4	44	C.O. 6	56	0.41	-13
17	C.O. 18	52	0.48	-4					
Predominantly myeloid marrows									
4	C.O. 4	69	0.37	-11	24	C.O. 6	61	0.42	-16
5	C.O. 5	68	0.43	-22	36	P.H. 3	62	0.57	-8
6	C.O. 3	63	0.35	-6	37	C.O. 4	71	0.33	-17
7	C.O. 3	62	0.48	-20	39	C.O. 5	69	0.55	-13
8	C.O.	68	0.56	-6	40	C.O. 7	67	0.77	-12
9	C.O. 5	73	0.72	-16	45	C.O. 4	66	0.51	-12
12	C.O. 4	61	0.69	-3	46	C.O. 12	65	0.54	-12

* Cu. mm. O₂ consumed/mgm. wet weight of tissue/hr., uncorrected for fat content of marrow.

† P.H. = acetylphenylhydrazine, C.O. = croton oil; for details see text.

cytes. The cells were washed once in Ringer solution and suspended in the same media used for the marrow experiments.

RESULTS. The experiments with marrow (table 1 and fig. 1) are divided into 3 groups on the basis of the cellular composition of the marrows. Normal rabbit bone marrow consists of approximately equal proportions of myeloid and erythroid cells (7); marrows containing from 40 to 60 per cent myeloid

cells may accordingly be designated a normal or intermediate group, being neither predominantly myeloid nor predominantly erythroid in composition. As indicated in table 1, the marrows in this group are almost entirely from animals that received a preliminary injection of croton oil intrapleurally. This uniformly causes a formation of exudate in the pleural cavity, but only about half the marrows, in the present series, were found to contain a preponderance of myeloid cells. In many instances this appeared to be due to a co-existing myeloid and erythroid metaplasia, the latter resulting, presumably, from a relative anoxia caused by impaired pulmonary ventilation. In other instances,

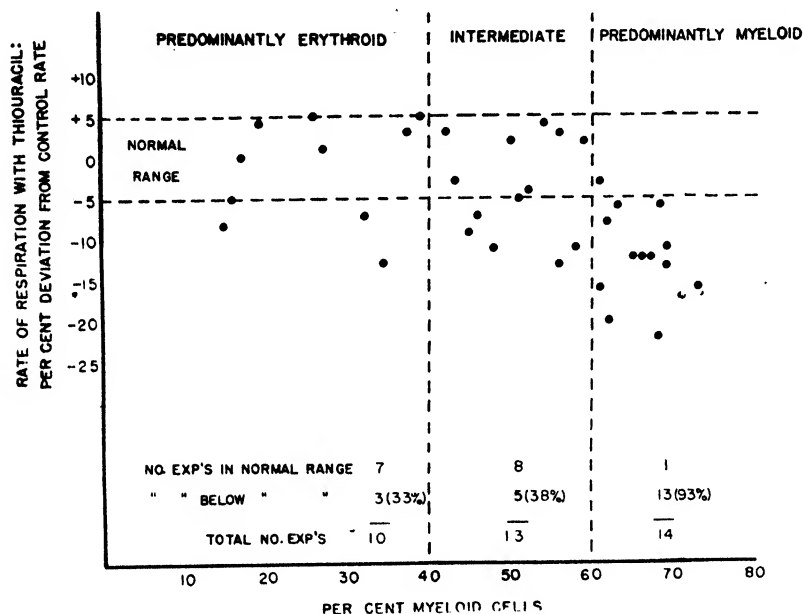


Fig. 1. Effect of thiouracil on respiration of rabbit bone marrow: influence of cellular composition of the marrow.

however, the lack of a predominating myeloid metaplasia had no obvious explanation.

The control Q_{O_2} values listed in the table vary over a considerable range, owing to their not having been corrected for the fat content of the marrows. In these experiments we are concerned with the relative rates of respiration (with and without thiouracil), and not with absolute values. Consequently, it has not seemed necessary to go through the procedures used in previous publications to express the results in terms of fat-free dry weight of tissue, and the Q -values in the table are only included for the sake of completeness.

Apart from these preliminary comments, the table and figure 1 would appear to be self-explanatory. Particularly by reference to the figure, it is seen that

thiouracil, in the concentration employed, has a variable but definite effect on marrow respiration—the higher the proportion of myeloid cells, the more consistent and the more marked the depressant effect on respiration. A variation of ± 5 per cent between the rates of respiration of the control and experimental samples is not regarded as significant and is so indicated in the figure. Only in the predominantly myeloid group of marrows does thiouracil exhibit a consistent, though small, depressant effect on respiration averaging -13 per cent, the P.E. of the mean being ± 1.3 per cent.

The greater sensitivity of the myeloid cells to this depressant action of the drug suggested that the action of thiouracil on the polymorphonuclear cells of rabbit peritoneal exudates should be studied. Four experiments similar to the above but using exudate cells instead of marrow, yielded depressions of respiration in the presence of thiouracil as follows: -11 per cent, -14 per cent, -15.5 per cent and -11 per cent. The average depression of respiration, -12.9 per cent ± 1.1 per cent, was the same as with the predominantly myeloid marrows. However, the cell counts of these marrows averaged only 66 per cent myeloid cells, and since the remaining erythroid cells have been shown to be considerably less susceptible to the action of the drug, the inference is that myeloid marrow cells (predominantly myelocytes) are more sensitive to the action of the drug than are the mature polymorphonuclear cells of exudates.

After the manometric experiments had been completed, samples of the vessel contents were placed on a warmed microscope stage for direct observations of the motility of the exudate cells and the myelocytes of the marrow. Comparisons of the motility exhibited in the control samples with that seen in the samples containing thiouracil did not reveal any obvious decrease in motility in the latter. This result is perhaps not surprising when it is recalled that the motility of leucocytes may be maintained under completely anaerobic conditions (12), but it is worthy of comment that the absence of a motility effect with thiouracil is in contrast to the situation with arsenite, which inhibits both respiration and motility out of proportion to effects on glycolysis (13).

The recent report of Cantor and Scott (5, 14) led us to attempt to protect the marrow from the depressant action of thiouracil by adding pyridoxine *in vitro*. This compound itself, however, was found to depress marrow respiration in concentrations greater than 10 mgm. per cent (5×10^{-4} M) and the depression was not antagonized by thiouracil. In 13 experiments in which this concentration was employed, no protective effect was observed against the depressant action of 100 mgm. per cent thiouracil (8×10^{-3} M). In interpreting these results, due allowance must be made for the discrepancy between the concentrations of the two compounds employed as well as for the obvious possibility that pyridoxine might act indirectly *in vivo* to counteract the effect of thiouracil without a direct antagonistic effect being demonstrable *in vitro*. Four preliminary experiments with liver extract also failed to disclose *in vitro* protective effects against the action of 100 mgm. per cent thiouracil.

DISCUSSION. While the point has been made that thiouracil appears to produce

a depression of respiration that is relatively selective for the myeloid cells, the data also suggest that in some instances the immature erythroid cells in the marrow may also be affected. In correlating this possibility with clinical cases exhibiting toxic effects of thiouracil, attention may be drawn to a case of fatal agranulocytosis reported by Gargill and Lesses (4), in which a generalized marrow hypoplasia was found. The absence of conspicuous anemia in such cases is explicable in terms of the long life of the erythrocyte relative to that of the polymorphonuclear leucocytes; the effects of transient hypoplasia of the erythroid elements in the marrow may easily pass unnoticed because either death or recovery occurs before the number of circulating erythrocytes is appreciably affected.

This discussion raises the question of the proper inferences to be drawn from the present *in vitro* experiments relative to the known toxic effects of thiouracil in patients. Clearly this subject must be approached with caution in view of 1, the species difference involved; 2, the high concentration of thiouracil used in these *in vitro* experiments³; 3, the relatively small depressions of respiration noted, and particularly 4, the obvious possibility that the mechanism of the toxic effects *in vivo* may be quite different from those demonstrated in acute experiments on isolated tissues *in vitro*. Nevertheless, it may be pointed out that there is a correspondence between *in vivo* and *in vitro* toxic effects in the following respects:

1. There is a considerable variation in the magnitude of the effects among different members of the same species. This variation is more striking *in vivo*, instances of depressant effects on the bone marrow of patients being the exception rather than the rule. However, in interpreting the present *in vitro* data, it should be noted that whereas, in the case of the predominantly myeloid marrows, a distinct depression of respiration is observed in 93 per cent of the marrows, a marked depression (of the order of 20 per cent or more) is found in only 2 out of 14 cases (14 per cent). It seems likely that definite impairment of marrow function would be evident only in this smaller group of cases, or might well require even larger effects on respiration. These considerations bring the *in vitro* and *in vivo* data into closer agreement than is at first apparent.

2. The myeloid cells, particularly the more immature ones in the marrow, are more seriously affected, and

3. The erythroid elements may be involved in a smaller number of instances.

These relationships consequently suggest that, whereas further data are clearly required to establish the true relationship between the *in vivo* and *in vitro* effects of thiouracil on bone marrow, it would seem warranted to grant, tentatively, a possible connection between the two effects. On this basis, the above methods furnish a means whereby chemotherapeutic agents may be tested for toxic effects on bone marrow before being released for clinical use. Similarly, the action of agents proposed to protect the marrow from depressant effects of drugs may also be studied.

* Cf. discussion of this point under "Methods".

SUMMARY AND CONCLUSIONS

The respiration of rabbit bone marrow and exudate cells *in vitro* is inhibited by thiouracil in 100 mgm. per cent concentration. The immature myeloid cells in the marrow are more consistently and strikingly affected, but the erythroid cells may also be involved. No effects on cell motility have been observed, and attempts to oppose the effect of thiouracil with pyridoxine have been unsuccessful. These methods might be employed to test new chemotherapeutic agents for possible toxic effects and also to test the action of compounds proposed to protect the marrow from depressant effects of drugs.

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THE DISTRIBUTION OF PHOSPHORUS COMPOUNDS IN THE GASTROCNEMIUS MUSCLE AS INFLUENCED BY THE AGEING PROCESS

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There has been a conspicuous lack of interest regarding the influence of ageing on the distribution of phosphorus compounds in muscle which is even more striking when one considers the fundamental rôle of phosphorus in muscular contraction. All of the large number of important advances in muscular chemistry have been concerned with the tissues of adult (?) animals with little recognition of the marked changes in muscular activity occurring during the total process of ageing.

Studies on the distribution of certain fractions of the total phosphorus content of muscle, such as the acid soluble compounds, have been investigated in a rather haphazard manner by a few workers. Cole and Kock (4) studied the phosphorus distribution in rat gastrocnemius muscle as influenced by age, diet, and irradiated ergosterol. Although not emphasized by the authors, their data showed a correlation between the distribution of phosphorus compounds and age in rats ranging in age from 21 to 168 days. Baldwin and Needham (2) in a study of the chick embryo, and Koschtozanz and Rjabinskaja (19) with rabbit embryo found phosphocreatine present in small amounts. Rapid increases in this compound were observed in the days following the hatching or birth of these animals. Fainchmidt, et al. (11) reported low values for phosphocreatine and adenosine triphosphate with corresponding high values for total phosphorus in rabbits 8 to 10 days old in comparison with adults. Several (28, 35, 36) inconclusive investigations have been reported on the changes with advancing age in the phosphorus fractions, especially the acid soluble phosphates, of various tissues of the rat.

METHODS. The albino rat is undoubtedly the most useful experimental animal available for the study of ageing. The exact day and even hour of birth can be readily ascertained. The nutritional requirements of this species are well defined, possessing the additional advantage of being very similar to those of man.

A colony of albino rats was maintained under uniform environmental conditions. The mean life expectancy of our animals was approximately two years. The stock diet consisted of Purina dog chow supplemented with green vegetables and powdered skim milk. Once a week cod liver oil, wheat germ oil, and brewer's yeast were also given. When animals reached desired ages, they were separated from their fellows and deprived of food but allowed water ad libitum for twelve hours before being anesthetized with nembutal injected

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intraperitoneally. In order to secure sufficient tissue for analysis from the younger animals, the muscles of several rats were pooled. Both gastrocnemii were carefully dissected away from surrounding structures, leaving circulation and nerves intact. After allowing at least 15 minutes for recovery from this manipulation, one muscle was frozen in situ in carbon dioxide snow. By this means twitching was reduced to a minimum. Since cooling of the muscle was relatively slow, circulation was maintained for some time, oxidative processes continued and values more physiologically representative for the resting organism were obtained.

The frozen gastrocnemius muscle was removed and analyzed for the various phosphate fractions by a modification of the procedure suggested by Eggleton and Eggleton (9). Phosphorus was determined by the method of Fiske and Subbarow (12) adapted to the Evelyn photoelectric colorimeter. Creatine was estimated by the method of Horvath (17).

RESULTS AND DISCUSSION. The four hundred and nine (409) rats on whom data were obtained were divided into twenty-six (26) groups. Since the greatest changes were observed in the early stages of ageing, the groups encompassing this period have a much narrower age range than the groups at later stages of life. If ten days of a rat's life be considered the equivalent of one year in the life of man, then our oldest group is comparable to approximately 80 years of age in man. The use of such an arbitrary chronological equivalent is not strictly accurate. Nevertheless, it offers us a guide which is comparatively more exact during the latter stages of life in both species. The data are presented in table 1 and figures 1 to 6. An illustration of the variations to be expected is shown in the data presented in table 2 on analyses performed on litter mates who were sacrificed at several ages. Analyses were also performed on the gastrocnemius muscles of two kittens, one and two days old, respectively. The values (not presented) are very similar to those observed in rats of about 12 days old, which is probably the equivalent physiological age.

1. *Total phosphorus.* It is uncertain what significance may be attached to this aggregate value, since the total phosphorus of muscle is a mixture of phosphates of diverse types and origins. The mean values for the total phosphorus of the gastrocnemius muscle of the rat increase within the first four weeks of life from 184 to a maximum of 262 mgm. per 100 grams of tissue. The steady decline during the remainder of the first year of life is followed by a fairly constant level of .224 mgm. throughout the remainder of the life span (table 1 and fig. 1). Lowry, et al. (21), whose observations began with the animal's sixth week of life, reported a downward trend of phosphorus with the ageing of the rat, but found no plateau in old age. Cuthbertson (7) found that the total phosphorus of adult muscle remains constant under a variety of conditions.

2. *Lipoidal or acid insoluble phosphorus.* Although the observed changes in total phosphorus are difficult to relate to specific physiological or biochemical phenomena, the various fractions of the total phosphates of muscle exhibit more characteristic patterns. The total phosphorus can be separated into two gross fractions because of their differential solubility in 4 to 5 per cent trichloroacetic

acid. The non-acid soluble portion contains phosphate compounds that are mainly lipoidal in nature. The acid-soluble phosphorus compounds are non-lipoidal and are chiefly associated with the muscle cells. It has been suggested that the physiological activity of a tissue is related to its phospholipid activity.

Figure 2 illustrates the changes that occur in the acid insoluble phosphates during the ageing process. The concentration of the acid insoluble phosphates

TABLE 1

The distribution of phosphates in the gastrocnemius muscle of the rat as influenced by the ageing process

(All concentrations are expressed as milligrams per cent in wet tissue)

MEAN AGE	NO. OF ANIMALS	TOTAL PHOSPHATE		ACID SOLUBLE PHOSPHATES		INORGANIC PHOSPHATE		CREATINE PHOSPHATE		ADENOSINE TRI-PHOSPHATE		HEXOSE PHOSPHATES		BARIUM INSOLUBLE PHOSPHATE		TOTAL CREATINE	
		Mean	P.E.**	Mean	P.E.**	Mean	P.E.**	Mean	P.E.**	Mean	P.E.**	Mean	P.E.**	Mean	P.E.**	Mean	P.E.**
days																	
3	23 (6)*	184		74		14		22		28		10		42		152	
12	32 (10)*	204	±0.4	98	±0.2	17	±1.4	28	±1.6	32	±3.0	12	±1.6	51	±2.7	181	±6.4
18	19 (11)*	227	±14.5	126	±1.3	23	±1.3	36	±1.6	37	±1.1	12	±1.7	75	±1.9	241	±7.3
23	21 (13)*	254	±3.9	124	±0.9	23	±0.3	40	±0.6	35	±4.0	15	±1.7	65	±1.5	307	±8.7
28	16	262	±3.0	137	±1.2	23	±1.7	44	±1.7	27	±2.3	22	±1.8	72	±1.8	315	±8.0
33	15	252	±3.2	146	±2.3	27	±1.3	49	±0.8	34	±1.8	21	±1.5	3	±1.7	321	±10.6
38	15	266	±3.3	148	±2.2	21	±1.4	57	±1.4	43	±1.9	18	±1.3	78	±1.8	326	±4.0
43	10	252	±1.8	145	±2.1	23	±0.9	49	±0.6	35	±1.3	19	±1.5	77	±1.6	360	±6.3
48	10	251	±1.6	149	±1.3	21	±0.7	51	±1.2	35	±1.7	24	±1.4	73	±1.9	384	±5.3
55	10	248	±1.7	151	±2.0	22	±1.1	58	±0.7	35	±1.3	23	±1.7	77	±1.9	388	±5.8
69	16	246	±1.0	151	±2.3	19	±0.7	56	±0.6	40	±1.1	19	±1.4	71	±2.8	396	±7.9
90	11	235	±2.9	153	±2.0	22	±1.0	59	±1.0	37	±1.3	20	±1.7	78	±1.8	381	±4.4
112	11	243	±2.6	156	±2.4	21	±1.1	55	±0.8	36	±1.2	19	±1.5	78	±1.7	404	±3.4
137	14	226	±1.1	158	±0.6	21	±0.4	60	±1.7	42	±0.8	18	±1.6	75	±1.7	434	±5.7
165	37	232	±1.1	155	±1.4	18	±0.9	62	±0.2	37	±1.0	16	±1.1	73	±1.2	412	±4.3
194	25	239	±1.2	154	±1.4	22	±0.8	61	±1.0	37	±1.0	20	±1.1	74	±1.2	421	±6.0
229	23	232	±0.5	151	±1.6	20	±0.7	62	±1.0	41	±1.2	18	±1.4	71	±1.9	422	±4.5
271	17	231	±1.5	155	±1.4	22	±0.8	58	±0.9	37	±1.2	20	±1.1	79	±1.5	423	±2.8
327	14	224	±2.0	156	±2.0	22	±1.0	60	±0.9	39	±1.1	12	±0.4	86	±1.2	418	±0.5
390	16	229	±1.3	156	±1.6	24	±0.9	59	±0.7	38	±1.6	16	±1.0	81	±1.3	420	±1.3
443	12	225	±2.5	152	±1.6	20	±0.8	59	±0.6	38	±1.0	20	±1.8	77	±1.6	409	±3.8
509	10	222	±1.1	154	±1.3	25	±1.1	57	±1.0	36	±2.4	20	±1.1	79	±1.5	421	±4.2
564	14	227	±2.6	157	±1.9	24	±1.0	59	±0.2	34	±1.1	19	±0.9	75	±1.7	428	±5.8
630	12	220	±1.2	147	±2.5	23	±0.8	57	±0.9	35	±1.0	17	±1.9	75	±1.6	401	±4.7
689	16	222	±1.7	147	±0.9	22	±0.7	58	±0.4	37	±1.1	16	±0.8	73	±1.7	409	±1.5
768	10	225	±3.2	146	±0.3	22	±0.8	61	±1.5	39	±1.6	14	±0.7	75	±1.1	397	±6.7

* Figure in parenthesis indicates number of groups into which animals were segregated to provide sufficient material for analysis.

** Probable error of the mean for small samples and unknown population (Pearl (23)).

is high even at the beginning of life, being some 60 per cent of the total phosphorus. The slight decrease in the amount of insoluble phosphorus which then occurs is followed by a rapid rise that persists for the remainder of the first month of the rat's life. However, since this rise is accompanied by a concomitant increase in both total and acid soluble phosphorus, the percentage remains constant at approximately 50. The return to low concentrations is more rapid than the previous rise, and the amount of acid insoluble phosphorus continues

to decrease to the adult level. There is some indication of stability during middle life at about one-third of the total phosphorus of muscle followed by another slight increase in concentration with the onset of old age.

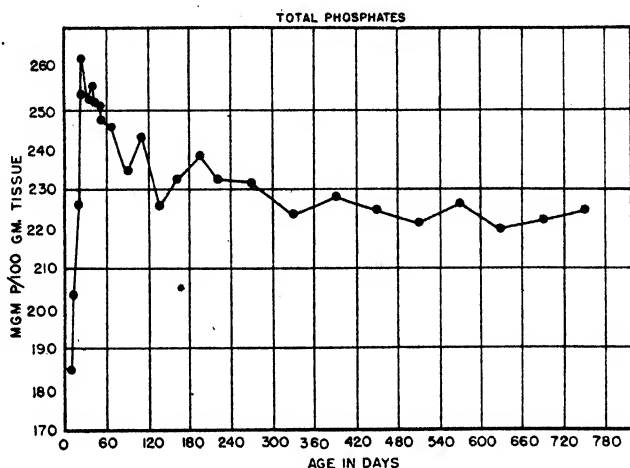


Fig. 1. The ageing process and the mean values, expressed in terms of phosphorus, of the total phosphates of the gastrocnemius of the albino rat.

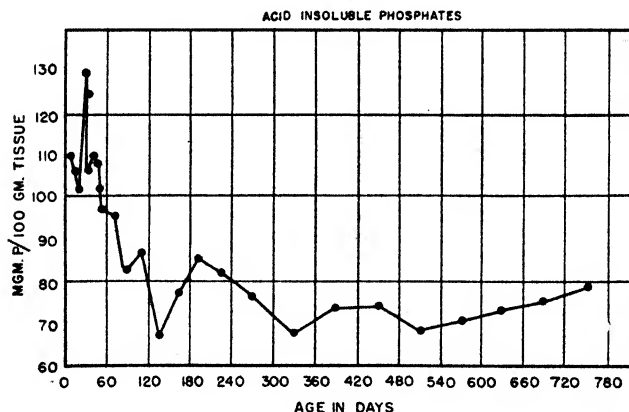


Fig. 2. Advancing age and the mean values for acid insoluble phosphates, in terms of phosphorus, of the gastrocnemius muscle of the albino rat.

Confirmation of our statement that the concentration of acid insoluble phosphate is altered by the ageing of the rat comes from Sinclair's observation (33) that the phospholipid content of the entire rat increases to a maximum at the third week of life and then slowly declines. No observations were made in old age. Our observations in old age imply an irreversible deposition of lipoidal

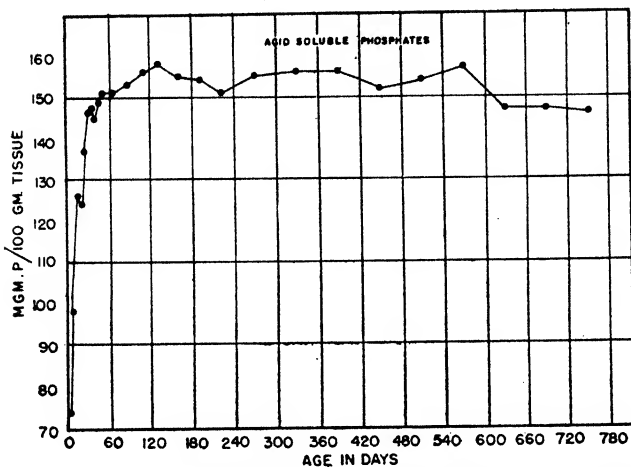


Fig. 3. Ageing and the mean values of the acid insoluble phosphates in the gastrocnemius muscle of the albino rat.

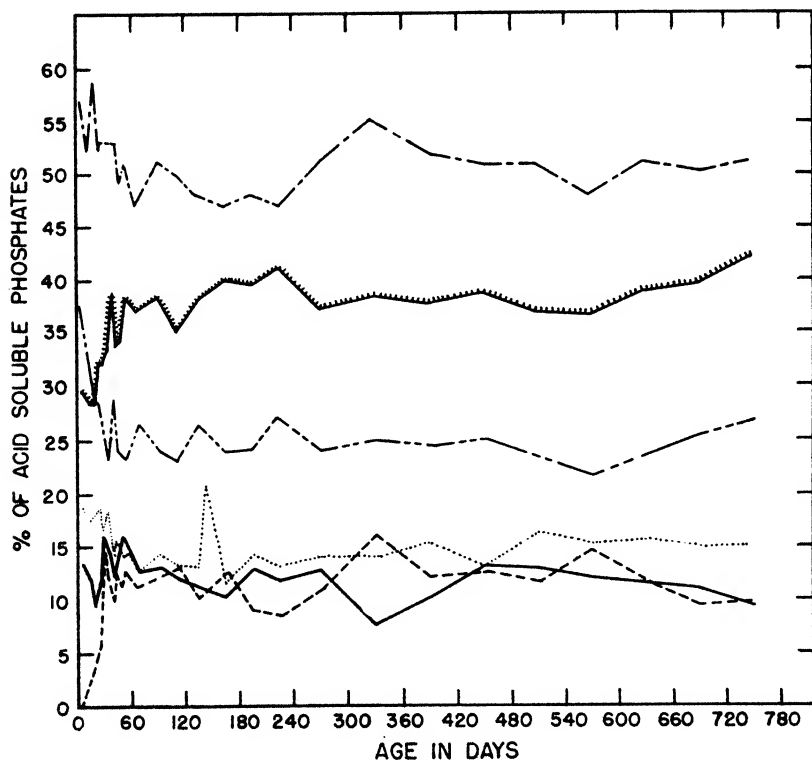


Fig. 4. The ageing process and the mean values of six fractions of the acid soluble phosphates expressed as per cents of the total and soluble phosphates in muscle.

material in the tissues due to changes in the rate of utilization and production of lipids as a phenomenon of ageing.

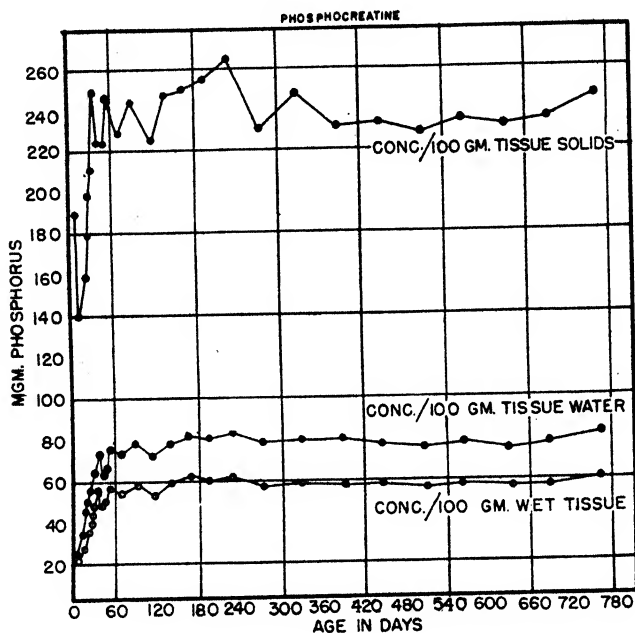


Fig. 5. Advancing age and the mean concentrations of phosphocreatine, as phosphorus, in the gastrocnemius muscle of the albino rat as found in 100 grams of wet tissue and calculated for 100 grams of tissue solids and for 100 grams of tissue water.

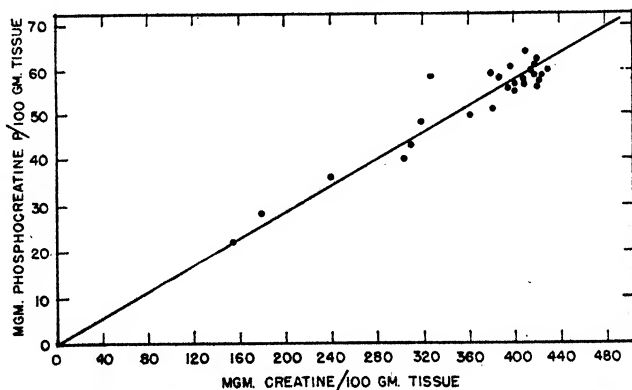


Fig. 6. A scatter diagram to illustrate the relationship between the phosphorus in phosphocreatine and the creatine in muscle at any given mean age throughout the life span of the albino rat.

3. *Acid soluble phosphates.* The other main fraction of the total phosphorus found in rat gastrocnemius muscle, the acid soluble component, presents an

entirely different picture with advancing age than does the acid insoluble fraction (fig. 3 and table 1). There is a rapid rise from 74 to 147 mgm. per 100 grams of tissue, a 100 per cent increase, in the first five weeks of life. This is followed by a slow rise to the adult average of 153 mgm. at about 100 days. Minor fluctuations occur thereafter. Cuthbertson (7) found that there was little change in the total acid soluble phosphorus of adult animals, even during muscular contraction. While there is a suggestion of a decreasing acid soluble phosphorus concentration in old age, it is not of sufficient magnitude (roughly 2 per cent) to be significant.

Struck et al. (35, 36) reported a striking effect of old age on the phosphorus compounds of rat muscle. It is interesting to compare their results on two groups of rats under similar dietary regimes but used for experimental purposes at different times. The following table presents this comparison; the group numbers are ours. An examination of these data reveals that their experiments were evidently poorly controlled. This is illustrated in their value of 125 mgm.

TABLE 2

Values for phosphate concentrations found at different ages in the gastrocnemius muscles of animals from two litters

(All concentrations are expressed in milligrams per cent in wet tissue)

AGE, DAYS (EACH ANIMAL).....	LITTER A				LITTER P					
	9	36	329	637	24	55	134	233	488	736
Total phosphates.....	221	276	245	215	241	253	235	226	225	223
Acid soluble phosphates.....	98	141	160	150	94	142	166	150	148	149
Barium insoluble phosphates.....	54	75	80	65	57	73	76	80	72	80
Inorganic phosphates.....	24	24	26	23	19	21	23	20	23	26
Creatine phosphate.....	28	48	59	55	32	57	62	59	51	56
Adenosine tri-phosphate.....	28	37	40	30	28	39	31	33	36	41
Hexose phosphates.....	13	17	22	14	10	30	27	21	25	13

for rats 90 days old in group 1 as compared to a value of 151 mgm. for rats of same age in group 2. This group of investigators has also reported a similar experiment, with the same general conclusion, on the tissues of man (28).

Age	Group 1	A.S.P.	Age	Group 2	A.S.P.
			28 days.....		136
			60 days.....		141
90 days.....		125	90 days.....		151
			120 days.....		158
			150 days.....		150
			180 days.....		143
300 days.....		155			
480 days.....		113			

4. *Ortho phosphorus*. It can be seen from the mean values and their small probable errors (table 1) that the orthophosphate concentrations in our animals were all within the ranges commonly accepted for resting animals under controlled experimental conditions. Our highest mean value of 27 mgm. is equal

to the average value of 27 mgm. found in resting muscle by Cori (6). That we were able to duplicate our experimental procedure throughout the entire study is shown in figure 4; the per cent of the acid soluble phosphorus that was broken down to form orthophosphate remained practically uniform at approximately 15 per cent.

The fact that a constant level of inorganic phosphate was found in all our animals cannot be taken as proof that changes consequent to age have not occurred. Defects in our experimental procedure may well have overshadowed any changes due to age.

5. *Hexosephosphates*. The hexosemonophosphates represent the earliest intermediary stage of carbohydrate metabolism since they are the compounds formed from the phosphorylation of glucose by the phosphate liberated during the breakdown of adenosine triphosphate. There is considerable doubt as to the actual concentration of hexosemonophosphate in muscle. The values in the literature vary from means of 10 to means of 20 mgm. per 100 grams of mammalian muscle tissue (3, 25).

Taking into account the variability that might occur in the hexosemonophosphates as the result of the breakdown or the resynthesis of adenosine triphosphate, there is an indication that the ageing process may have some influence. The average concentration of hexosephosphate in adult tissue is 19 mgm. per 100 grams of gastrocnemius muscle, but only 12 mgm. for animals in their first four weeks of life (table 1). This difference is not so striking when the changes with advancing age in the percentage of hexosephosphate phosphorus of the total acid soluble phosphorus are considered (fig. 4).

6. *Barium insoluble phosphates*. Before proceeding to the two compounds that are more intimately concerned with muscular contraction, it is necessary to clarify the somewhat ambiguous position of the barium insoluble phosphates. They represent roughly some 50 per cent of the acid soluble phosphorus during the entire portion of the ageing cycle that we studied (fig. 4). Analyses of this fraction, theoretically containing only ortho and nucleotide phosphates, have given extremely variable results (table 1) for reasons at present unknown. The inconstancy occurs in that portion of the fraction that is supposed to be nucleotide, and more specifically in the phosphorus remaining after two-thirds of the phosphorus of the nucleotide, the pyrophosphate, has been hydrolyzed. The equivalent of one-half of the pyrophosphate is left behind as ribose phosphate. Even when this amount is deducted and added to the pyrophosphate fraction to be classified as adenosine triphosphate, there is still left a small amount of phosphorus, the average value in the adult rat being 17 mgm. per 100 grams of muscle. As can be seen from figure 4, the concentration of this phosphate is much less in the developing rat, there being none during the first three days of life. There appears to be a rough and unexplained relation between the adenosine triphosphate and this unknown phosphorus; the percentage curves are practically mirror images of each other.

The calculation of this "unknown phosphate" is dependent upon the ashing of the barium insoluble fraction. It cannot be considered an error in the ashing

procedure, since there is such perfect agreement between the sum of the values obtained by ashing of both the barium soluble and barium insoluble fractions and that obtained from the ashing of the original trichloroacetic acid filtrate. Pollack, et al. (29) have called attention to a similar discrepancy in dog muscle.

7. *Adenosine triphosphate*. This is the key substance in the process involved in the carbohydrate breakdown in muscle. The concentration of this compound appears to be practically constant throughout life at about 38 mgm. phosphorus per 100 grams of muscle. If the percentage of ATP in the acid soluble phosphorus is plotted against age (fig. 4) it becomes evident that in the first days after birth the percentage of adenosine triphosphate, 38, is high. It then falls steadily during the first month of life, levelling off at approximately 25 per cent of the acid soluble phosphorus. That there is greater metabolic activity involving adenosine triphosphate in early life is further evidenced by the larger probable errors of the mean concentrations at that time. No other changes attributable to age are evident.

The relative constancy of the concentration of adenosine triphosphate throughout the life of the rat furnishes additional evidence of its importance. Whether we should limit it to its generally accepted rôle in muscle metabolism or whether it may have a more generalized function as a "middle man" for the easy transfer of phosphate to other phases of phosphorus metabolism is a problem that requires further study.

8. *Phosphocreatine*. Phosphocreatine plays a fundamental and significant part in the series of chemical events that occur during muscular contraction (9). Fiske and Subbarow (12) found a direct correlation between the presence of phosphocreatine and the ability of cat muscle to contract. Lundsgaard (22) also reported that the tension developed in frog muscles poisoned with iodoacetic acid is proportional to the breakdown of phosphocreatine.

It is common knowledge that the muscles of the very young animal cannot exert as great a tension as those of adult animals. A number of observations on the phosphocreatine content of the muscles of the embryonic, newborn and adult stages of life have been reported in the literature (2, 4, 11, 19). Our results confirm the findings that there is a difference in the concentrations of phosphocreatine in young and adult animals (table 1, fig. 5). However, the time curve of ageing shows that it is only during the first forty days of the rat's life that any changes occur; the concentration of the phosphorus in the phosphocreatine increases from 22 to 57 mgm. per 100 grams of tissue during this period. During adult life and into old age the concentration remains approximately constant at 60 mgm. of phosphorus. The small probable errors of the means reveal that these values do not vary greatly within the individual age groups.

The low values for phosphocreatine at birth and the gradual increase in its concentration up to and beyond the time of weaning can be explained as being a consequence of the development of an increasing and more vigorous state of activity on the part of the growing rat. However, the same explanation does not apply to the inactive aged animal whose muscle still has maximum amounts of the three major compounds essential to vigorous muscular contraction—60

mgm. of phosphocreatine, 38 mgm. of adenosine triphosphate, and roughly 500 mgm. of glycogen.

Since the time curve of ageing for creatine and phosphocreatine phosphorus have somewhat similar contours, curves were plotted of the changes in concentration of phosphocreatine calculated on the basis of 100 grams of muscle water and 100 grams of muscle solids (fig. 5). Curves similar to that plotted for the concentration of phosphocreatine in 100 grams of moist tissue were obtained.

The exact relationship of phosphorus and creatine in muscle is difficult to determine by direct analysis. Fiske and Subbarow (12), from their analysis of the compound they isolated from muscle, concluded that one mole of phosphorus and one mole of creatine are combined in phosphocreatine. The results of some workers suggest that 75 to 80 per cent of the creatine in voluntary muscle is in the form of phosphocreatine (23). However, Eggleton (8) from diffusion experiments with frog muscle suggested that only 65 per cent of the creatine is bound and that the rest is free. Gerard and Tupikova (14) reported that only 52 per cent of the creatine in the frog sartorius is bound.

Calculations of the ratio of phosphorus to creatine for a number of phosphate fractions in conjunction with the age of the rat were made (table 4). A positive correlation can be seen between creatine and all the phosphates for animals from young adults of approximately 110 days to aged rats of 770 days. This probably explains the correlation between acid soluble phosphorus and creatine that Myers and Mangun (26) emphasize as being so important. Their comparisons are made only on the basis of adult tissues. There is, according to calculations, only a single phosphorus compound, phosphocreatine, in which this correlation exists all through the life of the animal. The figures show an approximate constancy that is too striking to be accidental. According to these figures, some 60 per cent of the total muscle creatine is bound to phosphorus. The remainder, 40 per cent, is free or may be bound to some other component of muscle. A scatter diagram, figure 6, presents additional evidence of the relationship between phosphagen phosphorus and the creatine of muscle.

Let us assume that there is very little or no free orthophosphate (24, 34) within muscle cells and that therefore all of it that is estimated by our methods should be part of the phosphagen phosphorus. This assumption is not strictly valid since some of the orthophosphate may come from adenosine triphosphate or other phosphate compounds and some is normally to be found in the extracellular fluid. However, such an assumption permits the calculation of the ratio of this total phosphagen phosphorus to creatine (column 5, table 4). The ratio from 40 days to 770 days is practically constant and it is therefore possible that about 83 instead of 60 per cent of the creatine in the rat gastrocnemius is bound to phosphorus. This leaves only 17 per cent of the creatine uncombined. An entirely different ratio exists during the first 40 days of the rat's life. This ratio, roughly about one, accounts for all the creatine, leaving none in a free form. This is especially evident during the first three weeks of life. Accordingly, it is possible to assume that the limiting factor in the development of phosphocreatine is the available creatine. This would mean that the rapid

rise noted in phosphagen concentration during the same first 40 days of life is an artifact and that the phosphorylation of creatine is limited not by phosphorus, of which there is apparently an excess, but by the rate of synthesis and accumulation of creatine. This is an interesting possibility but it needs more experimental data to make it conclusive.

9. *Resumé of the acid soluble compounds in muscle.* The subdivisions of the acid soluble fraction of the total phosphate found in muscle include compounds of extreme importance for the performance of muscular activity. These compounds are adenosine triphosphate, phosphocreatine, hexosephosphates, orthophosphates, and some phosphates which are not yet definitely identified. Until

TABLE 3

Acid soluble phosphates in the muscles of adult mammals

(All values are expressed as milligrams of phosphorus per 100 grams of wet tissue)

REFERENCE	MAMMAL	ACID SOLUBLE PHOS- PHATES	INOR- GANIC PHOS- PHATE	PHOSPHO- CREATINE	ADENO- SINE TRI-PHOS- PHATE	HEXOSE PHOS- PHATES	UN- KNOWN PHOS- PHATES
Pollack, Flock, & Bollman (29).....	Dog	128	33	38	30	7	7
Sacks, Sacks, & Shaw (32)....	Cat	156	19	73	39		
Kerr & Blish (18).....	Domestic rabbit	161-196	23-43	39-66	36-50	10-21	
Milroy (25).....	Domestic rabbit	139	27	38	46	20	
Levine, Hechter, & Soskin (20).....	Albino rat	194	26	53	37	14	8**
Sacks & Sacks (31).....	Albino rat		17	65			
Cori & Cori (5).....	Albino rat	190	110*		64	9	7
Cori (6).....	Albino rat		27	59			
Cole & Kock (4).....	Albino rat	160	23	72			
Flock, Ingle & Bollman (13)...	Albino rat		29	45	34	11	
Hines & Knowlton (15).....	Albino rat	163	23	50	60		20
This author	Albino rat	153	21	59	38	19	17

* This represents the sum of the inorganic and phosphocreatine fractions.

** Hexose diphosphate.

now little has been known concerning the relative proportion of each and nothing of their distribution throughout the complete cycle of ageing in an organism.

In table 3 data which were obtained from analyses of adult muscles sampled under experimental conditions similar to ours, that is, muscles frozen in situ, were collected from the literature. Considerable discrepancy is evidenced even for animals which, like the rat, have been frequently used as experimental subjects. An interesting example of this is available in the data offered by the Coris (5, 6). In one series the average sum of the inorganic plus creatine phosphate is 110 and in the other series it is only 86. In an attempt to clarify the confusion regarding the distribution of the major components of the acid soluble phosphorus, the grand means of our analyses of the gastrocnemius muscles

of over 200 adult albino rats (ages 150 to 550 days) have been appended to table 3. Our values are approximately intermediate between the extremes in the data collected.

GENERAL DISCUSSION. This study was planned to establish a baseline of normality in relation to the age of an organism, in this case the albino rat, for those compounds having important rôles in the chemical events of muscular contraction. Our findings have shown that the most marked changes are observed during the earliest phases, that is, during the first forty days in the ageing of the organism. The variable analytical results obtained during the major portion of this period are rather unsatisfactory, but not surprising when one considers the rapid metabolic changes occurring at this time.

This picture of a rapid increase in concentration is exhibited by a number of components of muscle. However, this change is only transitory for two of these components of the gastrocnemius muscle. The highest concentration of total phosphorus and of acid insoluble phosphorus is reached at approximately the fourth week of life and then slowly decreases. This response is probably associated with Sinclair's (33) observation that the phospholipids of the entire carcass of the rat exhibit a similar change with advancing age (up to 100 days). Yannet and Darrow (38) have reported in their study of cats of two different ages that the muscle of the adult animal contains a greater concentration of phosphorus than does that of the young animal. While this discrepancy between their results and ours may be explained as being due to species differences, it is more probable that it was simply a question of sampling. Yannet and Darrow undoubtedly performed their analysis on the younger animals at the time the total muscle phosphorus was still increasing and had not reached the equivalent concentrations found in an adult animal. If the samples had been obtained when the kittens were slightly older, they might have found an identical or a greater concentration of phosphorus than was present in the adult cat.

The problems involved in evaluating physiological equivalents of ageing for chemical constituents of muscle are well illustrated by creatine. As an individual compound it reaches chemical maturity within 120 days, but in the form of its most important physiological combination, phosphocreatine, only 40 days are required to reach the state of chemical maturity. The striking manner in which one component of a tissue, in this case, creatine, by its slower rate of ageing² or growth can hinder the ageing of another, phosphocreatine, can be seen from table 4. If more creatine were available in early life, then phosphocreatine in all probability could be formed more rapidly and the radical differences in strength and endurance of young and adult animals would not be so great. In this respect it is interesting to note that Almquist and Meechi (1) found that the addition of creatine to the diet of the chick resulted in more rapid growth. This increased rate of growth was accompanied by increased

²The application of the term "ageing" to a component of muscle may not be strictly correct, but there is no reason to believe that ageing is the concern of tissue or organ alone. It is quite possible that the components of tissues or organs by their concentrations really determine the rate of ageing in the gross structure.

creatine in muscle. If creatine or its precursors were not available, muscular dystrophy resulted. On the basis of our findings and the observations of Almquist and Mecchi it can be tentatively proposed that muscular weakness is the result of interference with phosphocreatine synthesis as a consequence of deficiency in the supply of creatine. However, in mature rats the feeding of gelatin, a presumably creatinogenic substance, does not alter creatine concentrations in muscle (16).

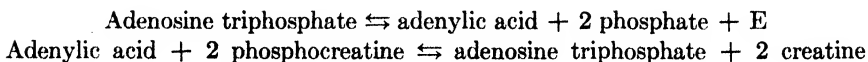
TABLE 4
The ratio of phosphorus to creatine during the ageing process*

MEAN AGE IN DAYS	TOTAL MUSCLE PHOSPHORUS TO CREATINE	ACID SOLUBLE PHOSPHORUS TO CREATINE	PHOSPHOCREATINE PLUS ORTHO PHOSPHORUS TO CREATINE	PHOSPHOCREATINE PLUS ORTHO PHOSPHORUS TO CREATINE	ORTHO PHOS- PHORUS TO CREATINE
3	5.1	2.1	0.61	1.00	0.39
12	4.8	2.3	0.65	1.04	0.40
18	4.0	2.2	0.65	1.04	0.40
23	3.5	1.7	0.55	0.87	0.32
28	3.5	1.8	0.59	0.90	0.31
33	3.3	1.9	0.64	1.00	0.36
38	3.3	1.9	0.74	1.01	0.27
43	2.9	1.7	0.57	0.84	0.27
48	2.8	1.6	0.56	0.79	0.23
55	2.7	1.6	0.63	0.87	0.24
69	2.6	1.6	0.60	0.80	0.20
90	2.6	1.7	0.65	0.89	0.24
112	2.5	1.6	0.58	0.80	0.22
137	2.2	1.5	0.58	0.79	0.21
165	2.4	1.6	0.64	0.83	0.19
194	2.4	1.5	0.61	0.82	0.22
229	2.3	1.5	0.62	0.81	0.20
271	2.3	1.5	0.58	0.80	0.22
327	2.2	1.6	0.61	0.83	0.22
390	2.3	1.6	0.59	0.83	0.24
443	2.3	1.6	0.61	0.82	0.21
509	2.2	1.5	0.57	0.82	0.25
564	2.2	1.5	0.58	0.82	0.24
630	2.3	1.5	0.60	0.84	0.24
689	2.3	1.5	0.60	0.83	0.23
768	2.4	1.6	0.65	0.88	0.23

* The ratio was determined from the mean values of phosphorus and creatine both expressed in millimoles per 1000 grams of moist tissue.

The relative constancy of the concentration of adenosine triphosphate throughout life is quite striking, suggesting the importance of this compound in intermediary metabolism. This is emphasized by the high values found during the earlier stages of ageing when adenosine triphosphate is expressed in terms of percentage of the acid soluble phosphates. Since it is highly efficient as a means of transfer of phosphate groups, its relatively high concentration in early youth may indicate that adenosine triphosphate has a rôle as a phosphate

carrier for other reactions than the esterification of carbohydrates. It is possible that it furnishes a phosphate group for the synthesis of phosphocreatine immediately on the formation of a creatine molecule, involving a reaction similar to that occurring during muscular activity. On the other hand it may simply signify a more prominent rôle of this compound in the supplying of immediate energy for muscular work during early life. According to the most recent scheme of the chemical events occurring during the muscular activity, two phosphorus containing compounds are primarily concerned with the initial stages of contraction, viz.:



The high concentration of adenosine triphosphate may mean that a portion serves as a reserve for protection from complete destruction as a result of strenuous muscular activity and yet allows for sufficient energy output. The low phosphocreatine content of muscle during this same period adds support to this "reserve" postulate. If it is true that the hydrolysis of adenosine triphosphate furnishes the immediate energy for contraction, it would follow that during severe work in the young animal a portion of the "reserve" must be used. Some of the adenylic acid probably is being deaminated to inosinic acid and this would also tend to lower the effective concentration of adenosine triphosphate.

According to our studies there are excellent biochemical conditions still present in the aged muscle for the performance of work. The findings of Robinson (30) indicated that men in the eighth decade of life have lost half of their capacity for transforming energy either anaerobically or aerobically. Therefore, the explanation of the decreased functional ability of muscle must be sought elsewhere.

The theory presented by Lowry (21) that there may be alterations in the efficiency of exchanges between blood and tissue due to the increased extracellular space in the muscle tissue of the aged is suggestive. On the other hand, alterations in the nervous regulation of muscle may be responsible for the diminished activity of the aged. The observations of Ellis (10) on the cerebellum suggest that the cause of dwindling muscular strength may be found in the governing mechanisms rather than the structure or composition of the muscle themselves. It is interesting to note that in denervated muscle (37) changes in contractility of the muscle can occur within a few minutes of denervation while chemical and histological changes may not appear for days and it may be weeks before the changes are striking.

CONCLUSIONS

The aged and the fully mature rats have essentially the same concentration in their gastrocnemii of those compounds that are intimately involved in the performance of muscular activity. It is only during the initial phases of the ageing process that changes are found. Large increases in the concentrations

of total phosphates, acid soluble and acid insoluble phosphates, hexosephosphates, phosphocreatine, and an unknown phosphate (in the barium insoluble fraction of the acid soluble phosphates) of muscle are observed within the first 30 days of life. This increase continues at a much slower pace for all of these components, except the total and acid insoluble phosphates, until constant adult levels are reached between 60 and 120 days. The changes with advancing age for total and acid insoluble phosphates are atypical. They reach their highest concentrations in 30 days but immediately begin to diminish, reaching stable values quite late, during the twentieth week of life. The acid insoluble phosphorus shows a slight tendency to rise again with the approach of senescence. A number of components of muscle show no changes in concentration attributable to the ageing of the organism. These include orthophosphate and adenosine triphosphate.

The influence of the ageing processes on adenosine triphosphate is not evident from a perusal of changes in concentrations. Expressed in terms of per cent of the acid soluble phosphates, adenosine triphosphate is high at birth and falls rapidly to adult levels within the first month of life. It is probable that adenosine triphosphate may have other rôles in the young animals' phosphate metabolism besides furnishing phosphate, with liberation of energy, for the esterification of carbohydrate during muscular activity.

Our knowledge of the acid soluble compounds of mammalian muscle which includes those most intimately involved in muscular contraction has been far from adequate. The data obtained during the course of this study are of value in clarifying the complex relationship of these compounds and furnish us with information regarding their distribution in muscle under resting aerobic conditions.

The slow development of chemical maturity by creatine of muscle appears to hinder the synthesis of phosphocreatine. This is borne out by calculations based on the assumption that there is no orthophosphate present in intact muscle cells. If no orthophosphate is present, then some 83 per cent of the creatine in the gastrocnemius muscle of an adult is bound to phosphorus. On the other hand, if orthophosphate is present in the concentrations observed, then only 60 per cent of muscle creatine is in the form of phosphocreatine and 40 per cent is free.

The waning muscular strength and the easy fatigability of senescence do not involve gross changes in the concentrations of those substances in muscle that are concerned with muscular contraction. The cause of loss of muscular power with advancing age must be sought elsewhere.

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BIOLOGICAL ENERGY TRANSFORMATIONS DURING SHOCK AS SHOWN BY BLOOD CHEMISTRY¹

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Enzyme studies have amply demonstrated that the rates of glycolysis and respiration are dependent upon the concentration of the breakdown products of ATP (adenosine triphosphate), and they have shown that the processes of glycolysis and respiration remove these breakdown products (1). The ATP system and the systems in equilibrium with it therefore actually form an over-all equilibrium system whose reaction constants form the basis of the homeostatic levels for the various compounds which are able to pass from the tissues to the blood and vice versa. Many of these compounds are in obvious equilibrium with each other, viz., lactic and pyruvic acids. In other cases the equilibrium is indirect, though well established, viz, blood glucose and inorganic phosphate. All four of the blood constituents named are in equilibrium with the A.T.P system in the tissues, and their levels in the blood are the resultant of the state of the tissues. In an animal in health and at rest the values for the compounds named remain quite constant. Even when performing work, the levels in the steady state usually do not deviate markedly from the resting values, owing to the remarkable capacity of the organism to mobilize energy when an increased demand is placed upon it. Only when 1, the stimulus is overwhelming, or 2, the recovery processes are interfered with, can any marked deviation from the homeostatic levels be expected.

In the case of the clinical syndrome recognized as shock, *one or both of these factors appear to be an invariable accompaniment of the shocked state*, and all of the methods for the production of experimental shock can be looked upon as variants of these two procedures for depleting the energy reservoirs. In other words, *they represent either an overwhelming stimulation, an interference with the process of energy mobilization, or a combination of the two.*

The present investigation attempts to determine whether or not the cycle of death may be *initiated* by a stimulation (e.g., injury, irritation, trauma, etc.) which produces a breakdown of ATP which exceeds the capacity of the organism to resynthesize this compound and thereby causes an accumulation of metabolic end products in amounts sufficient to set up the vicious cycle (see fig. 1).

The basis for the experimental testing of such a concept is somewhat as follows: If a standard type of stimulus could be shown to produce shock when admin-

¹The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Wisconsin.

istered at a certain intensity for a specified time, one could measure the changes in the concentration of various metabolites associated with the production of the shocked state. If graded sublethal amounts of the same stimulus were applied, one might expect to find chemical changes similar to those in the dying animals, but quantitatively smaller, and one could establish the *trend* of the changes associated with the stimulus when followed by 1, recovery, and 2, the development of shock. Furthermore, repeated sublethal stimuli might be expected to result in a hypertrophy of those portions of the organism which were put to the greatest stress, so that animals could be conditioned to withstand stimuli which would be lethal to unconditioned animals. And finally,

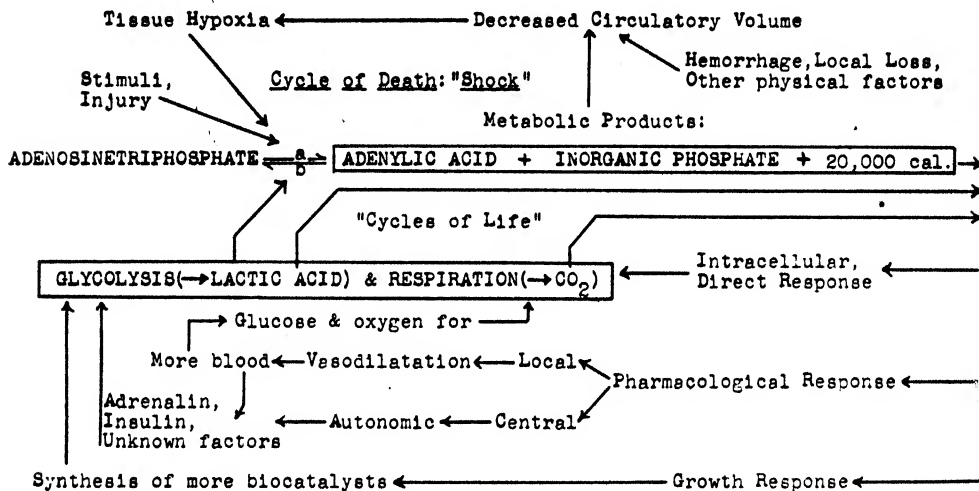


Fig. 1. The adenosine triphosphate concept of physiological organization in relation to the shock problem. Tissue hypoxia hinders the oxidative resynthesis of adenosine triphosphate (b) and therefore has the effect of shifting the adenosine triphosphate balance in the direction of breakdown (a). In the normal animal reaction (a) is thought to be one of the main sources of energy for function (1).

conditioned animals might be expected to show chemical changes qualitatively identical with those in dying animals, but quantitatively smaller.

As an example of shock produced by overwhelming stimulation we have studied the effect of trauma in the absence of anesthesia and accompanied by exercise and excitement using rats treated in the apparatus described by Noble and Collip (2). This is a rotating drum in which it is possible to administer trauma by repeated falls through a constant distance at a constant rate. The intensity factor is therefore constant and the duration of the stimulus can be empirically established so as to give the desired percentage of animals which develop shock. Furthermore, animals can be conditioned to withstand the shock-producing stimuli (3). The results of our experience with this method are reported elsewhere (4).

Of the utmost importance in a chemical study of shock is the selection of the compounds to be measured, the technique for the measurements, and the choice of material for analysis. In this study both blood and tissues were analysed. The blood may be expected to reflect changes in the metabolism of the tissues, particularly if the organism is stimulated at an intensity in excess of its ability to mobilize energy. Such an intensity of stimulation is implicit in the production of shock. If the ATP system is as intimately connected with cellular function as it appears to be (1) then evidences of its breakdown and of metabolically related reactions should be found in the blood; the analyses should be designed to reveal the products of these reactions.

The actual proof that ATP breakdown occurs requires an analysis of the tissues. The demonstration that glycogenolysis occurs is also dependent upon the analysis of the tissues. For death to occur, it is not necessary that every organ in the body shall have exhausted its energy reservoirs, since as soon as one vital organ dies, the others must follow. The blood analyses can reveal that the organism as a whole is exhausting its energy reservoirs but the tissue analyses are required to show the state of each specific organ. In interpreting the blood analyses which follow, we have examined the data in the light of the tissue analyses, which will be presented later (5). Since many vital tissues are too small or too diffuse to be analysed, one must attempt to deduce from the available tissue analyses and the blood analyses what constitute the limiting reactions in the response of the organism to shock-producing stimuli.

In addition to shock produced by over-stimulation our studies have also included the second main type of shock, which represents an interference with the processes of energy mobilization. Experimental shock based on an interference with the energy supply is usually produced either by decreasing the oxygen supply to one or more limbs by means of tourniquets, or by decreasing the oxygen supply to all the tissues by means of hemorrhage. The latter method was used by Long and associates (6, 7) in their studies on the blood chemistry of rats in shock.

We used the tourniquet method since it provided an opportunity to study the effect of complete ischemia on an isolated portion of the animal in terms of tissue analyses as well as blood analyses. The description of the method and its standardization will be presented elsewhere (4a). We believed that if the blood findings in the case of both of our methods for producing shock were in harmony with the blood findings in hemorrhagic shock (6, 7), one could begin to establish a general metabolic pattern for the shock syndrome. We believe that such has proved to be the case.

MATERIALS AND METHODS. Male rats weighing from 250 to 300 grams were obtained from Sprague-Dawley, Inc., and were kept in our laboratory at least five days before they were used for experimentation (4). The number of animals used for blood analyses was approximately two hundred.

Production of shock by the method of Noble and Collip. Rats were shocked by the rotating drum technique developed by Noble and Collip (2) using un-

conditioned and conditioned animals (3, 4). Four groups of animals were used as follows:

Group 1. Unconditioned animals not traumatized and used as controls.

Group 2. Unconditioned animals treated in the Noble-Collip apparatus for various periods and analysed at once or after a 30 minute rest period.

Group 3. Conditioned animals not traumatized and used as controls.

Group 4. Conditioned animals treated as in group 2.

The animals were traumatized for different periods of time as indicated in figure 2 to make possible the study of the trend of the blood substances during the period of trauma. Other animals were treated in the same way and allowed to rest for 30 minutes so as to determine the trend of the blood changes during recovery or during the development of fatal shock depending upon the amount of trauma induced. Survival studies were carried out on comparable rats during the same period and showed (4) that all the rats in group 4 (conditioned) would be expected to survive, while in group 2 (unconditioned), of those that received 13 minutes of trauma an average of only 10 per cent would be expected to live, and of those that received 4 and 8 minutes of trauma 100 and 66 per cent respectively would be expected to survive.

Production of shock by the application of tourniquets. Three groups of unconditioned animals were prepared for this aspect of the investigation. It is emphasized that the aim of this experiment was the study of the changing trend of the blood constituents during recovery of animals from a sublethal amount of ischemia induced by application of tourniquets, and during the development of fatal shock in animals that received a lethal amount of ischemia. The animals were nembutalized and treated by application of tourniquets (4a).

Group 1. Animals nembutalized for 4 hours without tourniquets and used as controls.

Group 2. Animals given sublethal amounts of shock by the application of tourniquets for 3 hours and 40 minutes. The blood was taken at different times after the removal of tourniquets as shown in figure 3. The survival rate was 47 per cent (4a).

Group 3. Animals given lethal amount of shock by the application of tourniquets for 4 hours. The blood was taken at the same periods as in the case of group 2. The survival rate was 3 per cent (4a).

Method of taking blood for analyses. Both the control and shocked rats were nembutalized immediately before the samples of blood were taken. Blood was drawn from the exposed abdominal aorta into an oiled syringe, and placed in a tube containing 30 mgm. of potassium oxalate, and 10 mgm. each of sodium fluoride and iodoacetic acid (8, 9, 10). Portions of the whole blood were added to the proper precipitating solutions within approximately two minutes after the blood was drawn. The remainder was centrifuged to obtain plasma for use in the determination of inorganic phosphate, pentose, and in certain cases, amino acid nitrogen.

The inorganic phosphate of the plasma was determined by the method of Fiske and Subbarow (11). The free and combined ribose was determined as

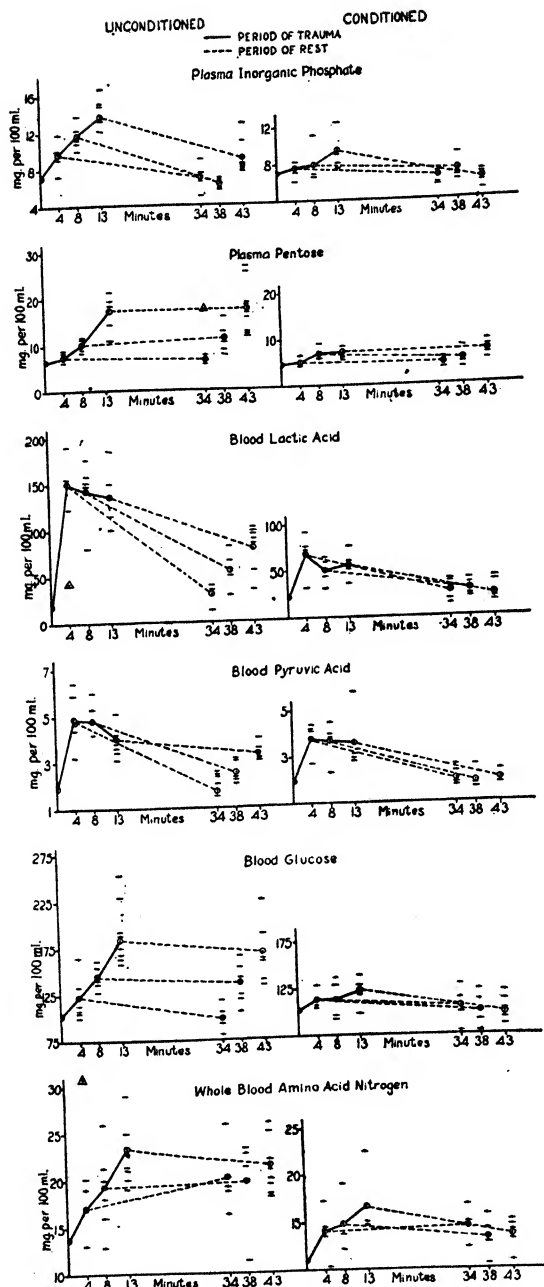


Fig. 2. Noble-Collip Shock. The analyses of the blood of unconditioned and conditioned rats shocked by the rotating drum method of Noble and Collip. The mean values of the different groups of animals were used, but the values of the individual determinations of each group are plotted to show the variation about the means. The method of Thompson (17) was used as the basis for the rejection of three outlying determinations. These three values are represented by Δ in the figure, and were not included in the respective means. The solid lines represent time in the drum, and the dashed lines represent a rest period of 30 minutes after being removed from the drum.

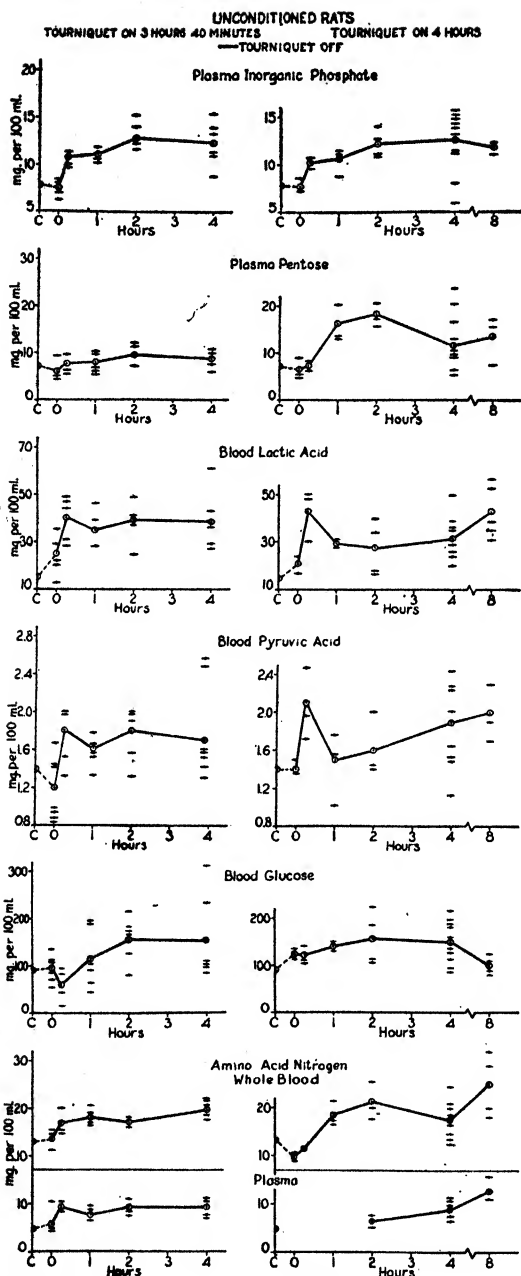


Fig. 3. Tourniquet Shock. The analyses of blood obtained from unconditioned rats at different times after the removal of tourniquets. In the case of the values for zero time, *O*, the blood was drawn immediately after the removal of tourniquets. In addition values are given for control animals, *C*, which were nembturalized for 4 hours without tourniquets.

total pentose by heating with orcinol in hydrochloric acid solution containing 0.1 per cent ferric chloride according to the method of Meijbaum (12), as recommended by LePage and Umbreit (13)². The method of Frame, Russell and Wilhelmi (14) was used in the determination of amino acid nitrogen. Blood glucose was determined by the method of Benedict (15), lactic acid by the method of Barker and Summerson (16), and pyruvic acid by the method of Bueding and Wortis (10).

RESULTS. The data presented in figure 2 are those obtained from the analysis of blood from unconditioned and conditioned rats shocked by the method of Noble and Collip, and the data presented in figure 3 are those obtained from unconditioned rats shocked by the application of tourniquets. The values of the individual determinations are plotted to show the variation about the mean values of the experimental data. The data as presented by these graphs show the trend of the blood changes during the application of the stimulus as well as the changes associated with the recovery or attempted recovery from the stimulus.

Rats shocked by the method of Noble and Collip. The results of the analyses reported in figure 2 show that during the period of the stimulus the increases in the blood constituents were much greater in the unconditioned than in the conditioned animals. The findings in this respect are in agreement with those reported by Neufeld et al. (18), but they have the additional value of showing the exact relationship of the findings to the period of stimulus and the period during which recovery took place or fatal shock developed.

During the period of stimulus the concentration of both inorganic phosphate and pentose increased in proportion to the duration of the stimulus, but there was a striking difference between the conditioned and the unconditioned rats. It is apparent that the conditioned animals had developed the ability to minimize the departure from the homeostatic levels of the circulating metabolites when a standard stimulus was applied. The fact that this increased ability to maintain the homeostatic level is correlated with survival value cannot be ascribed to a coincidence, since the increase in phosphate and pentose in the blood is correlated with the breakdown of ATP in the tissues, as shown by LePage (5).

It is of interest that the unconditioned rats leveled off at a lactic acid concentration which is close to the limit of the available bicarbonate of the blood, while the conditioned rats reached a steady state at a much lower level of lactic acid. It is particularly significant that the level of utilization of lactic acid in the unconditioned animals was not sufficient to keep pace with the continuing breakdown of ATP as judged by the outpouring of inorganic phosphate and pentose into the blood. In the conditioned animals, on the other hand, these products apparently did not escape from the tissues to such a marked extent, showing that in these animals the ability to oxidize pyruvate was at no time outpaced. The superior position of the conditioned animals is further borne

² It has subsequently been found that glucose interferes with the orcinol reaction to the extent of about 4 per cent. Since glucose was determined on all the blood samples it is possible to evaluate the pentose data in spite of this fact, and the variations in pentose are found not to be explainable on the basis of changes in the glucose concentration.

out by the low lactate:pyruvate ratios throughout the period of study in contrast to the very high ratios observed in the unconditioned animals (table 1).

The unconditioned rats had high concentrations of blood sugar in contrast to the conditioned rats, in which the increases in blood sugar were slight. The amino acid nitrogen increased to a greater extent in the unconditioned rats than in the conditioned animals.

The blood constituents of the conditioned animals returned to normal levels during the 30 minute period of rest. This was not the case with the unconditioned animals, particularly those of the group that received 13 minutes of stimulus, of which not more than 10 per cent would be expected to live. The unconditioned animals that received 4 and 8 minutes of trauma (from which approximately 100 and 66 per cent respectively would be expected to survive) were able to restore most of the blood constituents to near normal levels during the period of rest. They were in this respect similar to the conditioned animals that received up to 13 minutes of trauma.

Thus the animals which develop shock not only deviate further from the homeostatic levels following the stimulus, but they are *less capable* of restoring these levels during the rest period than are the animals which survive. That they are *not totally incapable* of removing the accumulated metabolites is explained on the basis of the well known fact that not all of the tissues die at once. The ability to restore the homeostatic levels is apparently destroyed in some vital tissue when the deviation from the normal level passes a critical point. The blood analyses reflect the resultant of the balance in all of the tissues, and if some tissues remain viable the levels of the circulating metabolites may tend to approach normal during the rest period in spite of the fact that a vital tissue is failing. The heart in shock retains its vitality until just prior to death of the animal, and since heart tissue appears to burn lactate preferentially, it would tend to lower blood lactate up to the point of death. According to this concept the animals in which fatal shock is developing need not show a complete loss of the ability to restore the homeostatic blood levels following excessive stimulation because some tissues may for a time be recovering while one or more other tissues are failing. The resultant blood picture can show improvement if the mass of the tissues which are recovering or remaining constant is greater than the mass of the tissues which are failing. This fact makes it necessary to analyse the tissues directly in order to assess their relative contributions to the total blood picture. This has been done in this investigation (5). Nevertheless the knowledge of the changes in the blood *during the period of stimulation*³ greatly facilitates the interpretation of the findings during attempted recovery.

Shock produced by application of tourniquets. The blood data are represented by the graphs of figure 3. In most instances the presence of the tourniquets had very little effect upon the concentration of the circulating metabolites as

³ It is manifestly impossible to secure these essential data from clinical cases or battle casualties, and the fact that the observations may be made while the lactic acid values are static or falling may have contributed to some of the confusion in this field.

compared to the values for the 4-hour nembutalized controls, and these in turn differed but slightly from the controls used in the Noble-Collip experiments (fig. 2). Following release of the tourniquets the accumulated breakdown products began to escape from the previously ischemic limbs, and changes began to take place in the blood.

In samples of blood taken immediately after the removal of the tourniquets the plasma inorganic phosphate was almost identical with the control values. During the next 15 minutes, the outpouring of metabolites from the released limbs resulted in a pronounced rise in both groups. Later there was a slight rise in both the sublethal and lethal groups but the levels remained essentially constant in both groups throughout the rest period. There was a marked rise

TABLE 1

Ratio of lactic to pyruvic acid in unconditioned and conditioned rats treated by the Noble-Collip method

TREATMENT*	L/P RATIO	
	Unconditioned	Conditioned
<i>min.</i>		
No treatment (controls)	9.9	11.9
4-0	31.9	18.0
4-30	17.6	13.8
8-0	29.6	14.0
8-30	22.2	15.7
13-0	33.8	15.7
13-30	24.0	14.1

* The first figure is the time in minutes during which the animals were shocked and the second figure is the time in minutes after the end of the period of shock at which the blood was taken for analyses.

TABLE 2

Ratio of lactic to pyruvic acid in unconditioned rats treated by the application of tourniquets

TIME AFTER REMOVAL OF TOURNIQUETS	L/P RATIO AFTER APPLICATION OF TOURNIQUET FOR:	
	3 hrs. 40 min.	4 hrs.
<i>hours</i>		
Control	10.3	10.6
0	20.7	14.7
$\frac{1}{2}$	22.3	20.5
1	21.6	21.1
2	21.7	17.2
4	22.4	16.7
8		21.6

in the pentose of the lethal group within one hour after release as compared to a slight rise in the sublethal group. These results are in harmony with the demonstrated breakdown of ATP in the ischemic muscle (5). Thus the higher level of pentose in the blood from the lethal group is correlated with a greater breakdown of the high energy phosphate ester.

The results of the tissue analyses (5) showed that the concentration of lactic acid in the ischemic muscle tissue was at a high level. This accounts for the steep rise in the blood lactic acid within 15 minutes after the tourniquets were removed.

The lactate:pyruvate ratios are given in table 2, and they show little difference in the two groups. However, in each case the ratio was elevated throughout the period studied, though to a level considerably below the maximum observed in the lethal group in the Noble-Collip experiments (table 1).

The constant level of lactic acid in the blood after one hour is ascribed to the acid from the ischemic muscles coming into equilibrium with the other organs which had been essentially normal up to this point. The levels of lactic acid attained in the steady state represent the balance between the formation by other tissues, the level of utilization and the amount released by the muscles. The fact that the liver and muscle glycogen of similarly shocked animals was depleted (5), and the fact that the level of lactic acid never returned to the normal value suggests that the utilization of lactic acid by the liver and heart never attained normal efficiency during the period of study. This subnormal efficiency must be a result of the fluid loss in the limbs, the products released from the limbs, or both, and will be considered in the discussion. The second rise in lactic acid at 8 hours is correlated with the development of irreversible shock and is analogous to the rise in lactic acid observed late in hemorrhagic shock (7).

The difference in the trends of the blood glucose during the first two hours suggests that the effective balance between adrenalin and insulin was not the same in the two groups, and this may have been an important factor in the ultimate outcome. The decrease in blood glucose at 8 hours in the fatal group is explained on the basis of decreased supply of carbohydrate reserves and is analogous to the fall in blood glucose in fasted hemorrhaged rats (6).

The rise in the amino acid nitrogen level soon after the tourniquets were removed was probably due to the outflow of amino acids from the ischemic tissue into the blood. The second rise that occurred in the lethal group after the tourniquets were off for four hours was likely a result of the increase in the breakdown of protein in the peripheral tissues, and the probable decreased ability of the liver to metabolize amino acids as suggested by Russell et al. (7) as the fatal stage of shock developed.

DISCUSSION. Although the present study is perhaps the first attempt to obtain simultaneously the values for inorganic phosphate, lactate, pyruvate, glucose, pentose and amino acid nitrogen in the blood of animals in the pre-shock, shock, recovery and irreversible shock stages, various other investigators have determined the concentration of one or more of the above constituents in various types of experimental shock, as well as shock in human beings. The findings parallel our own, when the same compounds were studied.

The most thorough analyses for inorganic phosphate appear to have been made by Duncan (19) who produced shock in dogs by three different methods (crush injury, hammer blows, and hemorrhage) and observed marked rises in each case. The rise in phosphate appeared to *precede* the development of shock in his experiments as it did in ours. Blitstein (20) also reported increases in inorganic phosphate in traumatic shock. We are not aware of any analyses for pentose in blood in shock, but increases in adenosine compounds have been reported (21) and Neufeld et al. (18) found an increase in uric acid in Noble-Collip shock.

The rise in amino acid nitrogen in the blood in hemorrhagic shock has been emphasized by Engel et al. (6) who established a definite inverse correlation

between blood pressure and amino acid nitrogen. We observed similar increases in amino acid nitrogen in both Noble-Collip and tourniquet shock. Neufeld et al. (18) observed mild rises in Noble-Collip shock.

The blood glucose has been reported to be high as well as low (6). The well known sympathetic hyperactivity associated with shock is undoubtedly responsible for elevated levels of blood sugar in shock in animals with adequate carbohydrate reserves. Late in shock (see fig. 3, 4 hr. tourniquets 8 hrs. after release) the carbohydrate reserves may begin to be exhausted with a resulting decrease in blood sugar. Engel et al. (6) used fasted or adrenomedullated animals and found hypoglycemia. Neufeld et al. (18) reported hyperglycemia in Noble-Collip shock.

The occurrence of acidosis in connection with shock was recognized by Cannon early in the study of this syndrome. More recently Gutmann et al. (22) reported a steep rise of plasma lactic acid as appearing to be a constant feature of traumatic shock. Subsequent work by Russell et al. (7) has emphasized the marked rises in lactic and pyruvic acids as shock progresses following hemorrhage. This was associated with increasing hypoxemia which depresses tissue oxidations and results in a glycolytic type of metabolism. In Noble-Collip shock, Neufeld et al. (18) reported high values for lactic acid and mild increases in pyruvic acid, following the stimulation, with decreasing values during the rest period. In their study the trends for lactic acid agree quite closely with those which we reported, but the values for pyruvic acid are considerably lower.

The results of the blood analyses reveal that shock is associated with an accumulation in the blood of the metabolites which result from the breakdown of the energy reservoirs. However, the sequence of events depends upon the method by which shock was produced. Three types of experimental shock will be considered in this discussion, namely, Noble-Collip, hemorrhagic, and tourniquet shock. The battle injuries which lead to the development of shock are seldom if ever perfectly analogous to any one of these three experimental methods of producing shock but since the results in the experimental types indicate that the underlying phenomena are fundamentally the same, it is likely that the conclusions derived from the experiments can be extrapolated to the syndrome resulting from battle injuries.

Noble-Collip shock represents an example of overwhelming stimulation, which depletes the energy reservoirs not by interfering with their resynthesis, but by a primary breakdown. In this situation the greater mass of tissue remains enzymatically functional and, following the stimulus, the concentration of end products actually shows a decrease throughout the period of observation. Moreover, the tissue analyses (5) show that all five of the major tissues studied progressively removed the end products during this period, so that the longer the animals remained alive, the lower was the concentration of metabolites such as lactic acid, not only in the blood but also in the five major tissues. Yet the correlation between survival and the speed of return toward normal as well as the magnitude of the original rise strongly suggests that the breakdown products are closely related to the development of shock, and that a tissue small in mass

failed during this period. The final events suggest that this tissue may have included the medullary centers.

On the other hand, shock may result from hemorrhage (6, 7). In this case, there is a progressive decrease in the oxygen supply to the tissues so that the resynthesis of ATP becomes increasingly ineffective. In this case the blood lactic acid is initially low and rises at a rate which must depend on the rate of hemorrhage. The vital tissues are protected as long as possible but eventually the oxidations are inadequate even in the medullary centers and in this case also the terminal event is respiratory failure and circulatory collapse, following a period of hyperactivity.

The events which follow the application of shock-producing tourniquets include many of the features of the other two types of shock. In the first place, the period of ischemia results in a local breakdown of energy reservoirs so overwhelming that when the 4-hour tourniquets are released the limbs never recover. The energy requirements for the maintenance of life in muscle cells cannot be met anaerobically for more than a short time. When the tourniquets are released, the body is flooded with the accumulated metabolites and a sudden rise in the concentration of these substances in the blood occurs. This is analogous to the similar rise observed in Noble-Collip shock, but is not so marked. In both cases, the recovery process begins and is most obvious in the case of lactic acid. That the energy reservoirs are still in a precarious state is suggested by the inorganic phosphate level, as well as by the other findings. As a result of the damage in the ischemic limbs, fluid is lost to these areas. This affects the animal in a manner analogous to hemorrhage, and as shock develops, the trend of the lactic acid, etc., goes through phases which vary from that seen in Noble-Collip animals to that which is characteristic of hemorrhage, and the animals die with rising values for these compounds. This is clearly illustrated in the 8-hour values following the release of the 4-hour tourniquets.

The development of shock following the release of tourniquets cannot be ascribed solely to fluid loss as is the case in experimental hemorrhagic shock. Haist and Hamilton (23) concluded that tourniquet shock was due in part to the release of muscle products since reapplication of tourniquets after the time of maximum swelling resulted in greatly decreased mortality. Similar observations are reported by Meyer and Shipley (24). Blood studies, to be reported later, show that the ability of the re-clamped animals to dispose of the metabolic end products is greatly improved. There is, therefore, a strong basis for concluding that the metabolic breakdown products from ischemic leg muscles can damage the enzyme mechanisms which ordinarily effect the synthesis of the energy reservoirs. These breakdown products are the same whether produced by incomplete synthesis (ischemic legs), or by overwhelming breakdown (Noble-Collip trauma). The problem is to discover whether the damaging products are among the known metabolites or whether they include unknown factors. Among the known products, the most obviously damaging agent is lactic acid, which is frequently produced in amounts nearly equivalent to the alkali reserve. The work of previous investigators (25) seemed to indicate that acidosis was not a primary factor in the development of shock. However,

the fact that lactic acid in the blood can decrease in the presence of developing shock may have contributed to this view (see fig. 2 and footnote 3). Our data are entirely compatible with the findings of Carey et al. (26) who have studied the pathology of the motor end plates in voluntary muscle. These workers found structural changes which were interpreted as a loss of the functional connection between nerves and muscles following hemorrhage, injections of lactic acid, and various other conditions.

The increased survival of conditioned rats, and the blood findings in these rats suggest that from a biochemical standpoint conditioning is largely the development of the ability to maintain the reservoirs of phosphate bond energy by oxidative mechanisms. The fact that some transfer of conditioning was effected (4) suggests that physically-hardened troops would be less likely to develop shock following critical amounts of injury.

From the therapeutic standpoint, one must consider the factors which are required for the effective utilization of oxidizable substrates. Enzyme studies carried out in conjunction with this investigation (27) as well as elsewhere, have revealed that the efficiency of oxidation (in terms of phosphate esterified) can vary widely *in vitro*, and there is some basis for suggesting that the efficiency may vary *in vivo*. That various hormones are involved in biological energy transformations is well known, but the exact rôle of adrenalin, insulin, cortin, thyroxin, and other hormones is as yet unknown. On the basis of the blood sugar data, it is possible to suggest that there may be a preponderance of adrenalin over insulin, and that in shock, insulin is either lacking in amount or operating under conditions which make it ineffective. The late decline in blood sugar values in the tourniquet animals suggests that the processes of gluconeogenesis are ineffective. It is well known that up to a certain point, restoration of blood volume is all that is required to bring about recovery from shock. However, if some vital humoral agent becomes depleted, it is apparent that restoration of blood volume will be inadequate, and one must attempt to discover whether the therapy should include one or several of the hormones which are involved in biological energy transformations. An examination of the status of the energy reservoirs during the period of therapy should aid in the critical evaluation of such therapy and provide information which is not available when survival is the sole criterion by which therapy is judged.

SUMMARY

1. Inorganic phosphate, pentose, glucose, amino acid nitrogen, and lactic and pyruvic acids were determined in the blood from unconditioned and conditioned rats shocked by the method of Noble and Collip, and from unconditioned rats shocked by the application of tourniquets.

2. Animals treated by the Noble-Collip method were studied during the period of stimulus and after a period of rest during which recovery began or fatal shock developed. The increases of the blood substances were greater in the unconditioned than in the conditioned animals, and the tendency to return toward normal levels was greater in the unconditioned rats that were expected to survive than in those that were expected to die.

3. The blood substances of the animals shocked by the application of tourniquets did not rise in certain cases to as high levels as did those of the Noble-Collip animals, but a more constant level was maintained. The changes in the concentration of pentose, glucose and amino acid nitrogen were different in the group of animals traumatized for 3 hours and 40 minutes as compared to the group traumatized for 4 hours by the application of tourniquets.

4. The changes in the concentration of the blood substances indicate that catabolic changes prevail in the tissues during the development of shock. The results obtained from the animals shocked by both methods are discussed from this standpoint. Although there are certain differences, it is believed that the results of the blood studies, regardless of the method used for producing shock, support this energy-depletion hypothesis.

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A COMPARISON OF THE NUTRITIVE VALUES OF DEXTROSE AND OF CORN SYRUPS AND OF THE EFFECTS PRODUCED ON THEIR UTILIZATION BY THIAMINE¹

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Our knowledge of the nutritive value of syrups and of the part played in their utilization by thiamine is still very limited. The present experiments were undertaken to supply some of this information for two corn syrups.

For these experiments the single food choice method was used. This method had been employed previously to study the nutritive values of a variety of carbohydrates, fats and proteins particularly of dextrose, sucrose and casein (1, 2, 3). Rats of a standard weight were placed on a diet in which one foodstuff constituted the sole source of nourishment. The length of time the rats survived and their energy or activity were taken as a measure of the nutritive value of the foodstuff. When a thiamine solution was offered in addition to the single food the effects produced on the survival time and energy were used to measure the effects produced by thiamine on the utilization of the foodstuff.

In the previous experiments it was found that on a single food choice of dextrose the rats survived on the average 37 days, but when given access also to a 0.02 per cent solution of thiamine hydrochloride they survived on the average exactly twice as long, 74 days. On dextrose alone the rats were very active, running 10 to 15 miles per day for the first 25 days on the diet, and on dextrose with thiamine their daily running activity averaged 10 to 15 miles as long as 60 days after the start of the single food diet. On sucrose alone the rats survived 37 days, just as long as on dextrose, and the rats were almost equally active. Access to thiamine solution increased the survival time to 56 days. On casein the rats survived on the average 33 days but were much less active than the rats on either dextrose or sucrose. Access to thiamine increased their survival time to 55 days. Thus, the results obtained with the single food choice technique gave a measure under the simplest conditions of the nutritive value of the various foodstuffs and of the remarkable effect which thiamine has on the utilization of these different foodstuffs.

This same technique has now been applied to the determination of the nutritive value of two corn syrups, Sweetose² and Special Corn Syrup³ and of the effects produced by thiamine on their utilization.

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METHODS. The individual all-metal cages used for these experiments have been described in detail in previous papers (1, 4). Each consisted of a revolving drum, 13 inches in diameter and 6 inches wide, a cyclometer which recorded revolutions of the drum in either direction, and a living compartment $3\frac{1}{2} \times 5\frac{1}{2} \times 11\frac{1}{8}$ inches with a non-spillable food cup and places for two 100 cc. and one 30 cc. graduated inverted bottles. A 3 inch hole in the partition which separated the two parts of the cage allowed the rats to pass freely between the drum and the living compartment.

The bottom of the living compartment was made of a $\frac{1}{2}$ inch wire mesh which permitted the feces to drop through to a sawdust filled pan several inches below, well out of reach of the rat. A space of approximately $\frac{1}{4}$ inch between the edge of the revolving drum and the central partition allowed the feces to drop through freely to a pan beyond reach of the rat. It was found that in almost all instances this type of cage eliminates coprophagy which, if present, may greatly affect the survival times and activity of the rats on the single food diets (1).

The cages were kept in a room the temperature of which was maintained as nearly constant as possible (between 75°-78°F.).

The rats were placed in the activity cages at an average age of 50 days and with weights that ranged between 80 and 100 grams. They were kept on the stock diet for the following 15 to 20 days. When they attained a body weight of 119 to 149 grams one of the syrups replaced the stock diet.

The two syrups were diluted with water so that each cubic centimeter of fluid contained 0.40 gram of syrup. (Actually each cubic centimeter of fluid contained only 0.33 gram of Sweetose and 0.32 gram of Special Corn Syrup, since water constituted 18.2 and 20.8 per cent of the syrups respectively.) This was done because of the difficulties involved in filling and cleaning the bottles when the straight syrups or higher concentrations of the syrups are used. Mixing the syrups with water might have complicated the results since with each cubic centimeter of syrup the rats were forced to take about three times as much water. In other words, they were not able strictly to make separate selections of the syrups and water. This shortcoming did not, however, turn out to affect the results.

Records were taken daily of the number of revolutions of the drums, the food and water intake, and of the vaginal smears. The rats were weighed weekly. The solutions of thiamine hydrochloride (in the 30 cc. bottle) and of the syrups

	¹ Sweetose (Staley Manufacturing Company, Decatur, Illinois)	² Special Corn Syrup (Corn Products Company, Argo, Illinois)
Dextrose	30.6	24.0
Maltose	27.9	20.0
Levulose (added)		6.0
Higher sugars	13.1	9.0
Dextrin	9.9	20.0
Water	18.2	20.8
Ash	0.3	0.2
	100.0	100.0

(in the 100 cc. bottles) were changed twice weekly. The rats were inspected at regular intervals for the presence of dermatitis and other pathological changes.

Twenty-eight females were used in these experiments, 13 for the Sweetose, 15 for Special Corn Syrup. Thirty-three rats from previous experiments on dextrose were used as controls. In so far as possible, rats were used which were nearly equally active before they started on the single food diets.

TABLE 1
Individual and average survival times on single food choice diets

CARBOHYDRATE	NUMBER OF RATS	SEX	AVERAGE AGE AT START OF SINGLE FOOD DIET	AVERAGE WEIGHT AT START OF SINGLE FOOD DIET	INDIVIDUAL SURVIVAL TIMES	AVERAGE SURVIVAL TIME
Without access to thiamine						
Special Corn Syrup.....	11	♀	days 64 (60-68)	grams 134 (124-142)	days 29, 33, 33, 34, 35, 35, 37, 38, 55, 56, 57	40
Sweetose.....	8	♀	63 (60-67)	138 (128-145)	29, 33, 40, 41, 42, 42, 45, 53	41
Dextrose.....	21	♀	64 (56-71)	137 (120-149)	28, 29, 32, 33, 33, 34, 35, 36, 36, 36, 37, 37, 38, 39, 39, 40, 40, 41, 42, 42, 54	37
With access to thiamine						
Special Corn Syrup + B ₁	4	♀	67 (67-68)	134 (134)	65, 79, 79, 79	76
Sweetose + B ₁	5	♀	69 (68-76)	138 (135-138)	61, 74, 76, 84, 96	78
Dextrose + B ₁	12	♀	62 (58-66)	139 (130-148)	62, 65, 67, 72, 73, 74, 74, 75, 76, 76, 82, 87	74

RESULTS. Table 1 summarizes the results. It gives the individual and average survival times for the rats on the single food choice diets of the two corn syrups without and with access to the 0.02 per cent solution of thiamine hydrochloride. For purposes of comparison records are shown also for rats on a single food choice diet of dextrose. Without access to thiamine the rats on Special Corn Syrup and Sweetose lived 40 to 41 days on the average, respectively, which is slightly longer than the average survival time of the rats

on dextrose (37 days). With access to thiamine the rats on the two corn syrups and dextrose lived almost exactly the same number of days; 76 days for the Special Corn Syrup, 78 days for Sweetose, and 74 days for dextrose.

Activity. Figure 1 summarizes the results. The ordinates show the average daily running activity in 10 day periods and the abscissae time in days. Records are given for 10 days on the stock diet and for the subsequent 10 day periods on the single food diets. Figure 1A shows that the activity of the rats on the Special Corn Syrup diet without access to thiamine increased from a daily

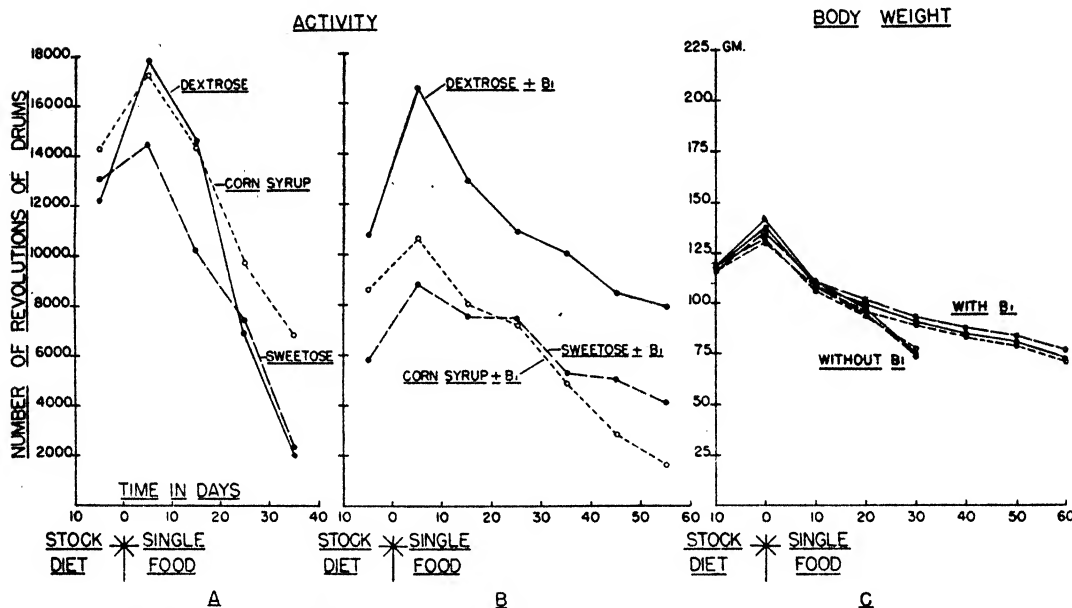


Fig. 1A. Graph showing average daily spontaneous running activity in 10 day periods as measured by revolutions of drum of rats kept on stock diet and then on a single food choice diet of syrups without access to thiamine.

1B. Same but with access to a 0.02 per cent solution of thiamine hydrochloride.

1C. Body weight curves for the rats on the single food choice diets of the two syrups and dextrose without and with access to the thiamine solution.

average of 14,200 revolutions for the 10 day period on the stock diet to an average of 17,300 revolutions during the first 10 day period on the single food diet. Thereafter the activity decreased at a constant and rapid rate. The activity of the rats on the Sweetose showed a smaller increase during the first 10 day periods on the single food diet but after that decreased at essentially the same rate. The activity of the rats on dextrose increased from 12,100 revolutions per day on the stock diet to 18,000 during the first 10 day period on the single food diet. Thereafter it decreased at a rapid rate. Considering the individual variations the results indicate that the rats were as active on either of the two corn syrups as on the dextrose.

Figure 1B shows the activity curves for the rats on the single food diet with access to the 0.02 per cent solution of thiamine hydrochloride. During the 10 day control period on the stock diet the group of rats which were later used for the syrup diets were not as active as those which were used for the dextrose diet. However, after the start of the syrup diets their activity curves have much the same shape. During the first 10 day period the activity of the rats on the syrups increased and during the successive 10 day periods decreased,

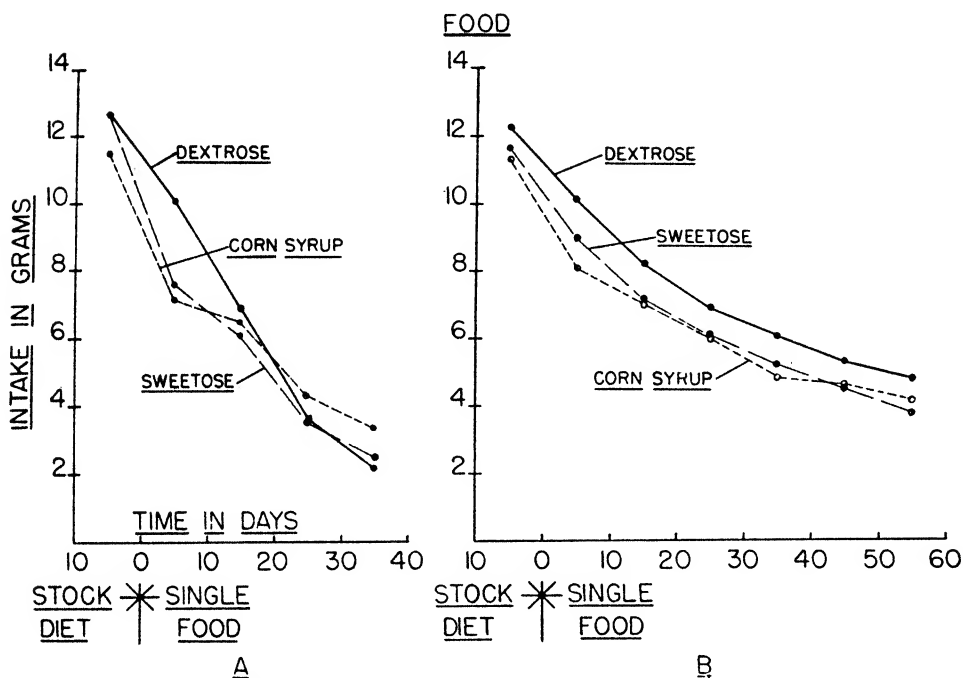


Fig. 2. Graph showing average daily food intake for the rats kept on the three single food choice diets (A) without access to thiamine (B) with access to the 0.02 per cent solution of thiamine hydrochloride.

but at a definitely slower rate than when they were on the single food diet without access to thiamine. When allowances are made for the difference in activity of the rats before they started on the single food diets the results indicate that the rats were as active on either of the two syrups as on dextrose.

Body weight. Figure 1C summarizes the results. The ordinates show body weight in grams; the abscissae time in days. The chart shows the curves for the rats on the single food without and with access to thiamine. Without access to thiamine the rats lost weight at almost exactly the same rate on the two sugars as on dextrose. With access to thiamine the rats lost weight at a slower rate than without the thiamine but the rate of loss of weight again was essentially the same for the rats on either of the two syrups or on dextrose. It will

be noted that at 60 days the rats with access to thiamine had almost exactly the same weight as the rats without access to thiamine had at 30 days.

Food intake. Figure 2 summarizes the observations made on the food intake. The ordinates give the intake in grams, the abscissae time in days. Figure 2A shows that on the single food diets both with and without access to thiamine the rats ate slightly less of the syrups than they did of dextrose. The difference, however, probably is not sufficiently great to be of any significance. The fact that the syrups were in the fluid form while dextrose was in the dry powdered form may account at least in part for these small differences in intake.

Water intake. Figure 3 summarizes the results of the observations made on water intake. In figure 3A the ordinates show the total intake in cubic centi-

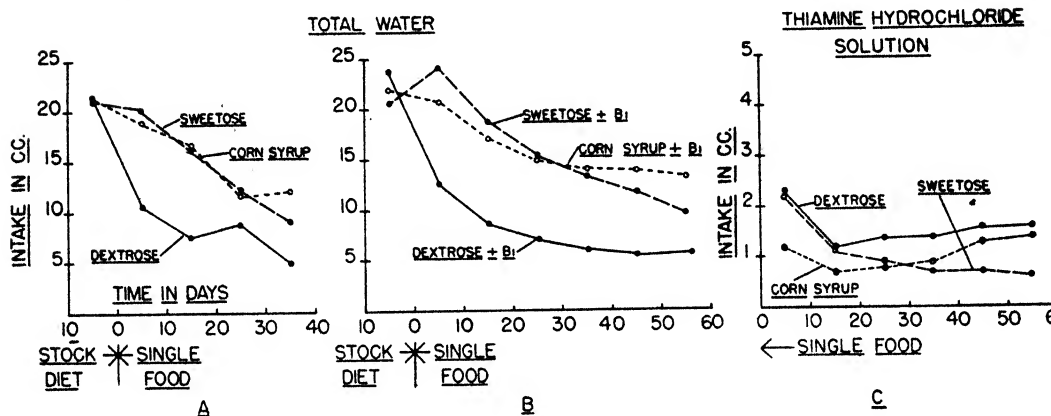


Fig. 3A. Graph showing average daily total water intake (plain water plus intake from syrup solutions) in 10 day periods for the rats kept on the single food choice diets without access to thiamine.

3B. Total water intake (plain water plus water from syrups and thiamine solutions) for rats with access to 0.02 per cent solution of thiamine hydrochloride.

3C. Total intake of the thiamine hydrochloride solution for the three groups of rats.

meters of water from the syrup mixtures and plain water, the abscissae time in days. No allowances have been made for evaporation, which makes a difference of approximately 1 cc. Practically all of the ingested water came from the syrup mixture, since the rats drank little or no plain water. Clearly the amount of water obtained in the syrup mixture was definitely more than the rats needed. Comparison with the water intake curve of the rats on dextrose would indicate that they received almost twice as much water as they needed. In figure 3B the ordinates show total water intake (water from the syrup mixture, the thiamine solution, and plain water). The rats on the syrup with access to the thiamine solution also drank more than twice as much water as did the comparable group of rats on dextrose. These rats were forced to take water not only with the syrup mixtures but with the thiamine solution as well.

Intake of the thiamine solution. Figure 3C summarizes the observations made

on the intake of the 0.02 per cent solution of thiamine hydrochloride. The ordinates give the intake in cubic centimeters. No allowances have been made for evaporation. The intake of the thiamine solution was essentially the same for the rats on the Special Corn Syrup as for those on dextrose. For both groups of rats it showed a steady increase, the lowest average occurring during the second 10 day period. The rats on Sweetose took slightly less of the solution and did not show the progressive increase during the last four 10 day periods.

Vaginal smears. The vaginal smears were essentially the same in the rats on the syrups as in the rats on dextrose. Most of the rats showed one four day estrus cycle after the start of the single food diet and then went into a state of constant diestrus.

DISCUSSION. The results of these observations show that the two corn syrups, Special Corn Syrup and Sweetose have essentially the same nutritive value as dextrose. Using the survival time as a measure they have the same nutritive value as other corn sugars and starches such as maltose (34 days), dextrin (36 days) and corn starch (31 days). In the absence of any data no comparison can be made with the cane or beet sugar syrups.

Thiamine has the same remarkable effect on the utilization of the corn syrups that it does on the dextrose and starch. For purposes of comparison it will be recalled that thiamine did not have as great an effect on rats kept on the single food diet of sucrose, or of casein. The intake of thiamine appeared to be essentially the same on the syrup diets as on the single food diets of other corn sugars. It averaged much below the amounts ingested by the rats on the single food diet of casein.

The rats seemed to like the corn syrups just as well as any of the corn sugars that have been tested. The inclusion of the 6 per cent of levulose to the special syrup did not seem to make this syrup more attractive.

SUMMARY

1. Eleven female rats of a standard age and weight survived 40 days on the average on a diet in which a corn syrup (Special Corn Syrup) constituted the sole source of nourishment. Eight rats on another corn syrup (Sweetose) survived 41 days on the average; 21 rats on dextrose survived 37 days on the average. The three groups of rats were equally active, lost weight at the same rate, ate approximately the same amount of food, and showed the same changes in the vaginal smears.

2. When given access to a 0.02 per cent solution of thiamine the rats of all three groups lived almost twice as long as on the single food alone; 76 days on Special Corn Syrup, 78 days on Sweetose, 74 days on dextrose. They showed essentially the same activity curves, lost weight at the same rate (not so rapid as without thiamine) and showed the same vaginal smear changes.

3. Both without and with access to thiamine the rats on the two syrups drank more water than did the rats on dextrose. The fact that they were forced to take water with their syrup in the mixture while the rats on the dry dextrose could take their water separately probably accounts for this higher water intake

4. It was concluded that the two corn syrups have essentially the same nutritive value as dextrose and that thiamine has the same effect on their utilization.

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NOTE ON THE EFFECT OF CAFFEINE ON AMMONIA AND UREA EXCRETION IN RABBITS

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The experiments of the early workers on the effect of caffeine on nitrogen excretion in the urine produced contradictory results (cf. 1). More recently, Means et al. (2) found an increase in the total nitrogen excreted by men given caffeine and Bachmann et al. (3) showed that creatine was excreted by men when moderate doses of the drug were taken. We have shown (1) that caffeine inhibits the formation of urea from ammonium salts by liver slices and we were therefore interested in determining whether a similar effect occurred in the intact animal.

EXPERIMENTAL. Male and female white rabbits weighing 2 to 2.5 kgm. were placed in individual metabolism cages and the urine collected every twenty four hours. Toluene was used as a preservative. The volume, pH, $\text{NH}_3\text{-N}$ and urea-N values were determined in each sample. The pH was measured by the glass electrode, the $\text{NH}_3\text{-N}$ by the permittit method, and the urea by the method of Ormsby (4). Some total nitrogen determinations were also done by the micro-Kjeldahl method. The rabbits were fed on oats and one large leaf of cabbage daily. This diet produced a urine with a pH between 5 and 6 which evidently represented the limit of the ability of the rabbit kidney to excrete acid, because the administration of ammonium chloride did not increase the acidity. The caffeine and the ammonium chloride were injected subcutaneously with sterile precautions twice daily, usually at 9 a.m. and at 5 p.m. Both substances were well tolerated by the animals for none showed any significant weight change during the experiment.

Table 1 summarizes the results on two male and two female rabbits. The caffeine caused some diuresis but it was not very great, and the pH of the urine was not altered. In all four animals an immediate rise in the $\text{NH}_3\text{-N}$ value occurred and was maintained as long as the caffeine was given. The effect on the urea excretion was not as great and the decrease was usually not evident until the second day of the caffeine administration. In rabbits 1 and 2 the urea excretion was very low on the first day of the subsequent normal period, and this may represent a delayed effect. When ammonium chloride was injected the volume and the pH remained unchanged, but the $\text{NH}_3\text{-N}$ and the urea-N increased markedly. It is interesting that the increase of urea-N is greater than can be accounted for by the nitrogen given in the ammonium chloride.

After three days of ammonium chloride alone, caffeine and ammonium chloride were injected together for three days. During this period there was some diuresis, no change in pH, a definite further increase of $\text{NH}_3\text{-N}$, and a large decrease in the urea output, so large in fact, that less urea was excreted under these conditions than in the normal urine. Rabbits 3 and 4 were also given glycine as a source of ammonia. Neither the $\text{NH}_3\text{-N}$ nor the urea-N were affected for the first two days, but on the third day a diuresis occurred and both values went up. When caffeine and glycine were given together a further increase in

TABLE 1

	MALE RABBIT 1				FEMALE RABBIT 2					MALE RABBIT 3				FEMALE RABBIT 4			
	Vol.	pH	$\text{NH}_3\text{-N}$	Urea-N	Vol.	pH	$\text{NH}_3\text{-N}$	Urea-N		Vol.	pH	$\text{NH}_3\text{-N}$	Urea-N	Vol.	pH	$\text{NH}_3\text{-N}$	Urea-N
	cc.		mg.	gm.	cc.		mg.	gm.		cc.		mg.	gm.	cc.		mg.	gm.
Normal	85		4.7	1.05	215		3.3	1.45	Normal	70	7.1	2.6	0.66	100	5.4	7.7	1.45
	120		4.4	1.15	90		3.9	1.35		60	5.6	3.5	0.88	40	5.3	5.4	1.58
	110		4.7	1.15	110		3.5	1.45		60	5.5	5.5	0.97	50	5.4	5.2	1.45
100 mgm. caffeine citrate injected twice daily	175		20.7	1.10	80		8.1	1.25	100 mgm. caffeine citrate injected twice daily	160	6.1	17.7	0.75	100	5.3	15.1	1.45
	90		9.5	0.49	125		5.5	0.30		180	5.3	18.1	0.60	110	5.3	26.3	0.90
	195	5.4	8.8	1.15	90	5.4	7.4	1.35		80	5.7	18.5	0.60	50	6.0	6.5	0.32
Normal	45	5.5	4.3	0.37	90	6.0	3.6	0.30	200 mgm. glycine injected twice daily	60	5.5	5.2	0.70	70	5.3	6.2	1.45
	120	5.5	4.7	1.45	115	5.6	3.6	1.35		60	5.3	5.0	0.80	70	5.1	4.9	1.45
										120	5.2	19.9	1.70	110	5.1	22.2	1.70
250 mgm. NH_4Cl (67 mgm. N) injected twice daily	90	5.7	9.9	1.70	110	5.5	9.4	1.25	200 mgm. glycine and 100 mgm. caffeine citrate injected twice daily	130	5.3	21.4	1.10	140	5.2	29.4	1.60
	140	5.7	11.9	2.35	80	5.2	11.0	1.90		170	6.0	22.5	0.65	170	5.4	29.5	0.80
	80	5.6	11.9	1.95	80	5.4	9.7	1.80		100	5.8	19.1	0.65	120	5.3	28.7	0.75
250 mgm. NH_4Cl and 100 mgm. caffeine citrate injected twice daily	210	5.6	19.1	0.70	220	5.2	17.9	1.00	Normal	100	5.7	6.7	0.90	80	5.3	8.2	1.35
	80	6.4	19.7	0.27	100	5.2	18.0	0.48		110	5.0	5.6	1.10	70	5.0	6.1	1.40
	160	5.8	18.4	0.75	160	5.3	16.8	0.85									
Normal	150	6.5	5.8	1.50	90	5.5	5.8	1.05	250 mgm. NH_4Cl (67 mgm. N) injected twice daily	110	5.7	9.1	1.25	150	6.0	10.9	1.70
	75	5.7	5.3	0.70	55	5.3	4.8	0.60		90	5.6	15.3	1.35	70	5.5	17.7	1.90
	80	5.5	4.2	1.45	90	5.3	4.3	1.05		100	5.2	16.8	1.25	50	5.3	18.7	1.65
									250 mgm. NH_4Cl and 100 mgm. caffeine citrate injected twice daily	110	6.1	22.2	0.55	150	5.5	26.2	0.70
										120	5.7	24.0	0.49	130	5.2	27.4	0.45
										130	5.3	21.4	0.60	120	5.2	24.7	0.65
									Normal	80	5.3	10.4	0.75	70	5.2	11.5	1.40
										60	5.2	5.9	1.25	60	5.2	7.6	1.71

the $\text{NH}_3\text{-N}$ occurred with an accompanying decrease in the urea. Both these effects are more evident in the female than in the male rabbit. Total nitrogen determinations show that whenever caffeine is given a rise occurs which is greater than can be accounted for by the rise in the $\text{NH}_3\text{-N}$ excretion and the nitrogen contained in the caffeine. It has not yet been determined whether this is creatine nitrogen, or some other nitrogenous substance.

DISCUSSION. These results with rabbits correlate with those obtained with caffeine and liver slices *in vitro*. Caffeine always causes some increase in ammonia excretion, but the decrease in urea excretion only becomes significant when,

apparently, the urea producing mechanism is overloaded by the injection of ammonium salts. Preliminary results obtained by Mr. R. O. Lipe show that caffeine increases the ammonia excretion in both men and women in the six hour period after taking 400 mgm. caffeine by mouth. In women the urea excretion is not affected, and in men there is a rise instead of a fall. Whether this sex difference will manifest itself after doses of ammonium chloride and caffeine remains to be seen. However, the results with animals show a satisfactory correlation between the *in vitro* and *in vivo* results. Such correlations are as yet the exception rather than the rule in the biochemical aspects of drug action.

SUMMARY

1. Caffeine injected into rabbits causes a slight diuresis, no change of the pH of the urine, an increase in the $\text{NH}_3\text{-N}$ excretion and some decrease in the urea-N excretion.

2. Injected ammonium chloride produces an increase in the $\text{NH}_3\text{-N}$ and urea-N excretion.

3. When ammonium chloride and caffeine are injected together a further increase of the $\text{NH}_3\text{-N}$ excretion occurs and there is a great decrease in the urea-N excretion.

4. Similar results are obtained when glycine is used as a source of ammonia instead of ammonium chloride.

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THE EFFECT OF VARIOUS STEROID HORMONES ON THE "ALKALINE" AND "ACID" PHOSPHATASES OF THE KIDNEY OF THE MOUSE¹

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In a previous report (1) it was demonstrated that testosterone propionate decreased the "alkaline" but increased the "acid" phosphatases of the kidney of the mouse; these changes coincided with the increase in size of the kidneys. There was no significant effect on the liver or intestine. In the meantime it has been shown that many other steroid hormones increase the size of the kidney of the mouse (2, 3, 4). It seemed, therefore, that a study of the phosphatases might provide information regarding the nature of the increase in kidney size observed with the different compounds.

PROCEDURE. *Animals.* Male mice² of the Murray-Little dba strain were castrated at 16.0 to 19.5 grams body weights; one month later pellets of the various steroids³ were implanted subcutaneously (2). The amount of steroid absorbed was increased by implanting two or more pellets and decreased by pellets composed of mixtures of the steroid hormone and cholesterol in the desired proportions (3, 5, 6).

Autopsy. The food, Purina Fox Chow checkers, was removed from the animal cages on the 29th day after implantation of the pellets and the mice were killed by decapitation on the 30th day. The kidneys were immediately removed, a slice was taken transversely through the mid-section of one of the kidneys for histo-phosphatase determination,⁴ the remainder was weighed and dropped into a heavy walled tube containing 5 ml. of redistilled water. The kidney was homogenized (1), the mixture diluted with redistilled water to 40 ml. per gram tissue, 3 per cent by volume of redistilled sulfur free toluene was added and the mixture allowed to autolyse in the refrigerator at 35-40°C. The phosphatases were determined within 10 days⁵ after autopsy.

¹This investigation was aided by grants from the Josiah Macy Jr. Foundation.

²The mice were provided by the Biological Station, Springville, N. Y. through the courtesy of S. G. Warner.

³The steroids were provided by Ciba Pharmaceutical Products Inc. through the courtesy of Drs. E. Oppenheimer and C. R. Scholz. Many of these compounds were specially prepared for this and related studies. The testosterone propionate was provided as Perandren and the 17-methyl testosterone as Metandren.

⁴These results will be published later.

⁵The homogenized mixture may be kept in the refrigerator for 21 days or more without any significant changes in enzyme activity. The "alkaline" is more stable than the "acid" phosphatase. The autolysis, however, is complete within 24 hours or less.

Determination of phosphatases. The "alkaline" phosphatase was determined at pH 9.8 by the King-Armstrong method (7) with minor modifications. The homogenisate was diluted 15 times with redistilled water just before use, 0.5 ml. of this dilution was added to 10 ml. of the substrate mixture of disodium phenylphosphate buffered with sodium barbital and incubated for one hour at 37°C. Then 4.5 ml. of the Folin-Ciocalteu reagent was added, the tubes placed in the refrigerator for 1 to 2 hours and the mixture was filtered. Five milliliters of the filtrate was pipetted into a test tube (15 x 125 mm.), 5 ml. of 8 per cent sodium carbonate added and the color which developed in 1 to 2 hours was determined with a Klett-Summerson photo electric colorimeter with a no. 66 filter. Blank determinations were run simultaneously by omitting the incubation period. Standard phenol solutions were always run at the same time as the color determinations were made on the tissue samples.

The "acid" phosphatase was determined on 0.5 ml. of the stock homogenized mixture as above but with the Robinson-Gutman substrate, disodium phenylphosphate and citrate buffer (8), set at pH 5.4.⁶

RESULTS. *Castration.* As in the previous study (1) castration does not affect the total amount of "alkaline" phosphatase present in the kidney but increases the amount per gram of kidney due to a decrease in size of this organ. On the other hand, there is a decrease in "acid" phosphatase which is most significant in the total amount.

Steroid hormones. All of the steroids that increase the size of the kidneys produce a similar effect on the phosphatases (table 1). The "alkaline" phosphatase is decreased both in total amount and per gram of tissue while the "acid" phosphatase is increased. In both instances the changes are associated with the change in kidney size. The graphic (fig. 1) presentation of the results obtained with testosterone not only illustrates these changes but also gives the range of separate determinations.

DISCUSSION. The similar results obtained with all of the steroids indicates that these hormones have a similar effect on the metabolic processes influenced by these two phosphatases. This, however, is not true of all physiological effects produced by these substances. The 17-methyl compounds, 17-methyl testosterone and 17-methyl androstenediol-3 α , 17 α , enhance the increase in arginase activity obtained in the kidneys of the mouse (9) and the creatine metabolism in man (10, 11).

The very great decrease of the "alkaline" phosphatase both in total amount and per gram of tissue indicates that not only is the kidney increasing its size in every instance by forming non-"alkaline" phosphatase containing tissue but also is decreasing the amount that is normally produced. Apparently the kidney's need for this enzyme is decreased. The increase of acid phosphatase on the other hand suggests that either more of the tissue which produces this enzyme is being deposited in the kidney or else the cells that produce this enzyme

⁶In the previous study (1) the pH 4.9 recommended by Robinson and Gutman (8) for blood was used. Investigation of the effect of pH showed that the optimum pH for tissue was between 5.2 to 5.6 with a slight peak at 5.4.

TABLE 1

The effect of various steroids on the "alkaline" and "acid" phosphatases of the kidney of the mouse

TREATMENT	NO. MICE	STEROID AB- SORBED	KIDNEY	"ALKALINE" PHOSPHATASE		"ACID" PHOSPHATASE	
				Total per cent change*	Per gram per cent change*	Total per cent change*	Per gram per cent change*
		mgm.	mgm.				
Normal.....	12		414	-4	-42	+85	+13
Testosterone.....	4	0.14	389	-10	-41	+62	+9
Testosterone.....	4	0.37	417	-38	-58	+69	+6
Testosterone.....	4	1.15	505	-53	-75	+100	+6
Testosterone.....	7	4.06	469	-49	-50	+96	+8
Testosterone.....	1	8.5	477	-58	-77	+92	+7
Testosterone.....	3	16.2	541	-59	-81	+148	+13
Testosterone Propionate†.....	4	0.20	392	-28	-52	+38	-9
Testosterone Propionate†.....	4	0.88	458	-48	-71	+77	+1
Testosterone Propionate†.....	5	2.33	490	-35	-70	+96	+2
Testosterone Propionate†.....	1	4.10	446	-75	-85	+85	+8
17-Methyl Testosterone†.....	4	0.14	415	-6	-44	+77	+5
17-Methyl Testosterone†.....	4	0.57	424	-37	-62	+77	+9
17-Methyl Testosterone†.....	4	1.30	466	-58	-76	+92	+6
17-Methyl Testosterone†.....	6	4.26	507	-55	-77	+92	-2
17-Methyl Testosterone†.....	1	8.10	540	-62	-82	+123	+7
17-Methyl Testosterone†.....	4	14.40	525	-65	-83	+119	+8
17-Ethynyl Testosterone§.....	2	1.75	257	-13	-14	+4	+1
Androstanol-17 α , one-3.....	5	0.65	424	-36	-61	+42	-14
Androstanol-17 α , one-3.....	2	6.4	518	-54	-77	+88	-1
Androstanol-17 α , one-3.....	4	7.1	505	-62	-80	+88	-2
Δ^4 Androstenedione-3,17.....	2	19.7	420	-46	-66	+62	+1
Androstanediol-3 α ,17 α	3	0.25	383	-14	-37	+15	-18
Androstanediol-3 α ,17 α	1	1.5	470	-30	-60	+96	+9
Androstanediol-3 α ,17 α	4	3.8	472	-52	-73	+73	-6
Androstanediol-3 α ,17 α	4	5.5	464	-55	-75	+89	+4
Androstanediol-3 α ,17 α	3	5.9	466	-64	-80	+127	+25
Androstanediol-3 α ,17 α ,Acetate-3.....	6	2.3	421	-13	-47	+65	+1
Androstanediol-3 α ,17 α ,Acetate-3.....	8	3.0	399	-41	-62	+46	-6
Androstanediol-3 β ,17 α	4	2.0	333	-14	-30	-4	-23
17-Methyl androstanediol-3 α ,17 α	4	0.6	459	-53	-73	+65	-7
17-Methyl androstanediol 3 α ,17 α	5	3.2	517	-53	-77	+81	-8
17-Methyl androstanediol 3 α ,17 α	6	5.5	448	-62	-78	+58	-8
17-Methyl androstanediol 3 α ,17 α	5	6.9	507	-65	-82	+135	+21
17-Methyl androstanediol-3 β ,17 α	7	1.8	420	-27	-55	+65	+3
17-Methyl Δ^4 -Androstenediol-3 β ,17 α	4	2.4	347	+13	-18	+39	+3
Δ^5 -Pregnenol-3 β , one-20.....	1	4.6	305	+18	0	+19	+3

* Change from average values of 8 castrated mice. "Alkaline" phosphatase: Total Units 118, per gram 456; "Acid" phosphatase: Total Units 2.6, per gram 10.0.

† Perandren.

‡ Metandren.

§ Pregnenynolone, Lutocylol.

have increased their production in proportion to the increase in kidney size. The latter possibility is the more likely (12).

The effect of the steroid hormones on the phosphatases differs with species. Testosterone and testosterone propionate increase the "alkaline" phosphatase in the kidney of the castrated (13) and adrenalectomized rat (14). The "acid" phosphatase and also the kidney weight are not significantly affected in these animals.

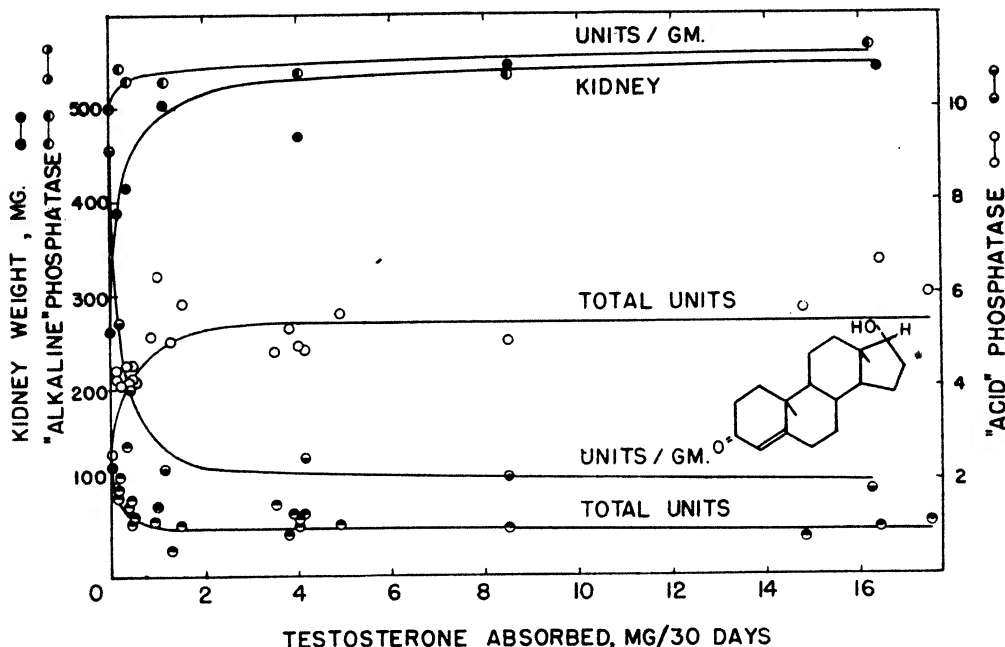


Fig. 1 The effect of testosterone on the "alkaline" and "acid" phosphatases of the kidney of the mouse. The values for the total units of both enzymes are plotted separately but the values for the castrated animals, the units per gram of kidney and kidney weight are plotted as averages.

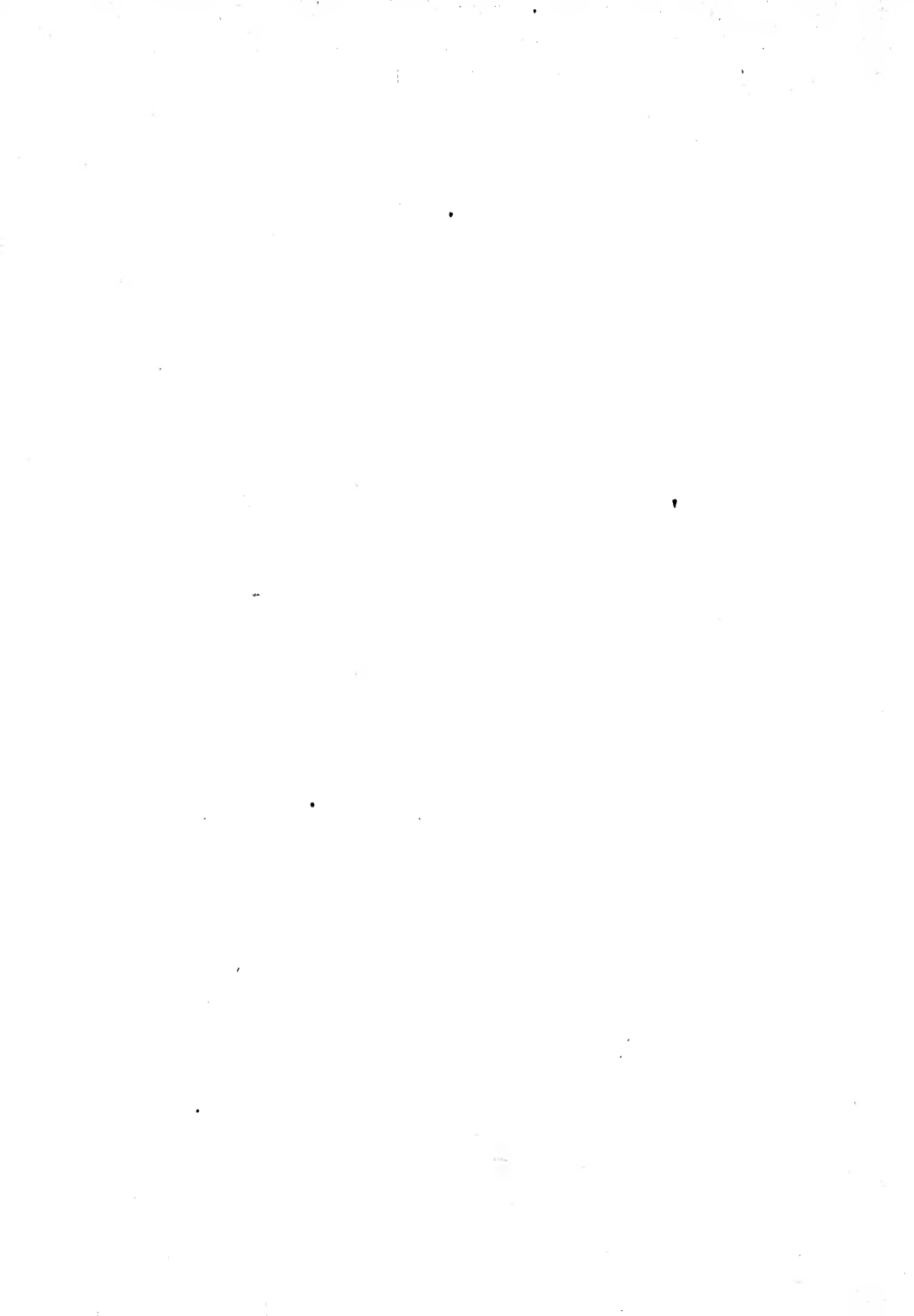
SUMMARY

The "alkaline", pH 9.8, and the "acid", pH 5.4, phosphatases of the kidneys of castrated mice were determined after treatment for 30 days with 21 different steroids implanted subcutaneously as pellets at different dose levels. Similar results were obtained with all of the compounds. As the kidneys increased in size, the "alkaline" phosphatase decreased both in total and per gram of tissue. The "acid" phosphatase, on the other hand, increased in total amount but remained the same or slightly increased per gram of tissue.

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THE INFLUENCE OF SODIUM LAURYL SULFATE ON THE BIOLOGIC RESPONSE TO THE GONADOTROPINS AND TO INSULIN

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In studying the denaturation of the protein hormones (1), there was included in the list of denaturants the detergent, sodium lauryl sulfate (S.L.S.), which has been investigated rather extensively in relation to the simple proteins (2). The results of the first trial (exp. I), rather unexpectedly, indicated an increase instead of a decrease in biologic activity. In early studies we have shown that an increase in biologic activity of the chorionic and pituitary gonadotropins may be brought about by divided dosage or its equivalent delayed resorption (3, 4, 5, 6). The response of the mare serum hormone is affected neither by divided dosage nor delayed resorption. The results of the present study show that S.L.S. may be added to the list of preparations which are effective in delaying resorption, and that it, therefore, markedly augments the effect of the pituitary hormone and has no influence upon the response to the mare serum hormone. The interest which is attached to its action is, as will be shown, in that it does not function by forming an insoluble preparation but rather by forming a depot of fluid, which is but slowly resorbed. In the case of insulin, the detergent in the concentration used effectively to delay the resorption of the gonadotropin, produced a decrease in the activity of the insulin through its *in vivo* destruction. Insulin denatured by S.L.S. *in vitro* retained its full activity when administered in low S.L.S. concentration.

EXPERIMENTAL. The sheep pituitary and chorionic (prolan) gonadotropins were stable powders prepared in this laboratory by fractional alcohol precipitation. The hormone from mare serum was the highly purified clinical preparation of the Cutter Laboratory. Immature female rats were used in the assay of the gonadotropins. The assay procedures for the mare serum and chorionic gonadotropins were recently described in detail (1), the statistical treatment depending upon the comparison of the response in litter mates. In experiments I to IIIa, pairs of 9 litter mates were employed per experiment. The assay procedures for the sheep pituitary hormone are given in table 2. The assay reference curve when copper is used to delay resorption and effect the maximum response has been described (1) and is used in experiments IX and X to estimate recovery of hormone.

Iletin (Eli Lilly and Company) was used as the source of insulin. Seven

experiments with insulin were performed in all. In 3 experiments (table 3, expts. CS, GH and IJ) insulin was incubated at 38°C., in 5 per cent S.L.S. concentration for 1.5 hours at pH 8.2, for 3 hours at pH 6.0, or for 24 hours at pH 8.0. The solution for assay was prepared by dilution so that the S.L.S. concentration was reduced to 0.12 per cent and the insulin concentration to U 2.5. The assay of the resulting solution was performed by the method of Lacey (7) in which the experimental material is compared with a standard insulin by cross matching in rabbits and recovery is calculated on the assumption

TABLE 1

Influence of sodium lauryl sulfate upon the response of immature female rats to chorionic (prolan) and pregnant mare serum gonadotropins

HORMONE AND EXPT. NO.	TREATMENT	NO. OF RATS PER ASSAY	MEAN ORGAN WEIGHT IN ASSAY	RECOVERY OF HORMONE	CORRECT ASSAY RANGE 19 OF 20 TIMES
			mgm.	%	%
I. Prolan	400 I. U. per cc., 1 hr. at 37.5°C. in 1% NaCl, pH 7.4, then dilution and addition of 0.07% S. L.S.	9	80 (u)		
	400 I. U. per cc., 1 hr. at 37.5°C. in 5% S.L.S. pH 7.4, then dilution to 0.07% S.L.S.	9	88 (u)	112	12
II. Prolan	Control assay solution in 1% Na Cl	9	63 (u)		
	Assay solution in 0.45% S.L.S.	9	79 (u)	120	7
III. Mare serum	Control assay solution in 1% Na Cl	9	99 (o)		
	Assay solution in 1.1% S.L.S.	9	98 (o)	100	11
IIIa. Mare serum	Control assay solution in 1% Na Cl	8	113 (o)		
	125 U. per 0.5 cc. in 5% S.L.S. 2 hr. at 38°C.; assay solution diluted to 0.25% S.L.S.	8	115 (o)	100	17

(u) uterine weight; (o) ovarian weight.

tion that the mean drop in blood sugar over a 5-hour period is proportional to the dose. In two experiments (AB and OP) insulin was administered in a 0.5 per cent S.L.S. solution and in two other experiments (VW and XY) in a 1 per cent S.L.S. solution. In these experiments three assays were based on the method of Lacey. In the other experiment (AB), the insulin was administered in doses of 1 U per kilo and blood sugar determinations were performed 3, 6 and 9 hours after administration. Ten to 12 rabbits were used per assay in all experiments.

RESULTS. It was apparent from the results of experiment III, table 1, that S.L.S. had no influence upon the response to the mare serum hormone. Incubation in a 5 per cent solution for 2 hours at 38°C. resulted in no loss of activity (expt. IIIa). In contrast chorionic gonadotropin in the presence of S.L.S.

TABLE 2

Influence of sodium lauryl sulfate upon the response of immature female rats to sheep pituitary gonadotropin

EXPT. NO.	TREATMENT* OF HORMONE ADMINISTERED IN ASSAY	MEAN OVARIAN WEIGHT	MEAN UTERINE WEIGHT	NO. OF RATS PER ASSAY
IV	Hormone in 1% NaCl, pH 7.0	13 \pm 0.8	18 \pm 1.5	7
	Hormone in 0.1% S.L.S., pH 7.0	17 \pm 1.3	29 \pm 4.2	7
V	Hormone in 1% NaCl, pH 7.0	14 \pm 0.9		7
	Hormone in 0.45% S.L.S., pH 7.0	45 \pm 8		7
VI	Control, no injection	12 \pm 0.8	16 \pm 1	6
	Injection of 4.5 mgm. S.L.S. in 1 cc. vol., pH 7.0	11 \pm 0.5	15 \pm 1	6
	Injection of 4.5 mgm. S.L.S. in 1 cc. vol., pH 7.0, injection of hormone at site removed	13 \pm 0.7	21 \pm 3	6
VII	Hormone in 1% NaCl, pH 7.0	13 \pm 0.8	15 \pm 1	6
	Hormone in 1% S.L.S., pH 7.0	77 \pm 10	84 \pm 6	6
VIII	Hormone in 1% NaCl, pH 4.0 (soluble)	17 \pm 0.6	22 \pm 2.5	7
	1 mgm. hormone + 0.3 mgm. S.L.S., pH 4.0 (precipitate)	18 \pm 1	28 \pm 2.5	7
IX	Precipitate of 1 mgm. hormone, 0.4 mgm. S. L. S., pH 4.0, in 0.26 cc. vol.	12 \pm 0.6	18 \pm 0.7	6
	Filtrate of above, assayed with copper	30 \pm 4	85 \pm 7	6
X	Precipitate of 1 mgm. hormone, 0.3 mgm. S.L.S., pH 4.0, in 0.6 cc. vol. assayed with Cu	36 \pm 5		6
	Filtrate of above assayed with copper	33 \pm 4		6
XI	Hormone in 0.5% S.L.S. solution	41 \pm 4.5		8
	1% hormone in 5% S.L.S. solution 1 hr. at 38°C., then diluted to 0.5% S.L.S. concentration	39 \pm 3.5		8

*The total dose per rat was 1 mgm. in 1 cc. vol. given in 4 equal doses on 4 days, or the equivalent of 1 mgm. of precipitate or filtrate.

showed a significant increase in activity (expts. I, II). One milligram of the sheep pituitary hormone given subcutaneously once daily in saline produces doubtfully significant increases in ovarian and uterine weights (see expts. IV, VI and VIII). The addition of increasing concentrations of S.L.S. viz., 0.1 per cent, 0.45 per cent and 1.0 per cent, under the same conditions of assay

produced an increase in ovarian response, the highest concentration of S.L.S., producing a mean increase of 65 mgm. in ovarian weight. The low concentration increased the ovarian weight 3 mgm., the uterine weight 11 mgm. Furthermore, incubation of the hormone in 5 per cent S.L.S. concentration at 38°C. for 1 hour produced no loss in activity (expt. XI). It has previously been shown

TABLE 3

The in vivo and in vitro effect of S.L.S. on insulin as measured by mean drop in blood sugar in rabbits as per cent of fasting value for 1.5, 3, and 5 hours and ratio of experimental/control values so obtained

EXPT. NO.	NO. OF RABBITS	TREATMENT OF INSULIN	DROP IN BLOOD SUGAR. MEAN + STD. DEV. MEAN	RATIO OF EXPERIMENTAL TO CONTROL DROP IN BLOOD SUGAR
			%	%
C	10	Control, heat at pH 8.6, 1.5 hrs., at 38°C., dilute to U 2.5 conc.	32 ± 2.1	S/C = 97 ± 9
S	10	Heat in 5% S.L.S., pH 8.2 for 1.5 hrs. at 38°C.*	31 ± 2.9	
G	11	Control pH 2.0, U 2.5	32 ± 1.7	H/G = 99 ± 6
H	11	Heat in 5% S.L.S., pH 6.0 for 3 hrs. at 38°C.*	31 ± 2.4	
I	11	Control pH 7.0, U 2.5	37 ± 2.2	J/I = 99 ± 3
J	11	Heat in 5% S.L.S., pH 8.0 for 24 hrs. at 38°C.*	37 ± 2.0	
O	12	Control pH 2.0, U 2.5	38 ± 2.8	P/O = 82 ± 6
P	12	0.5% S.L.S., pH 6.0, U 2.5	31 ± 2.8	
V	12	Control pH 2.0, U 2.5	36 ± 1.8	W/V = 45 ± 5
W	12	1.0% S.L.S., pH 6.0, U 2.5	16 ± 2.3	
X	12	Control pH 2.0, U 2.5	29 ± 2.4	Y/X = 65 ± 5
Y	12	1% S.L.S., pH 6.0, U 2.5 (crystalline insulin)	19 ± 1.7	

* 0.1 per cent S.L.S. concentration after dilution in solution used for assay. Insulin concentration U 2.5.

that administration of the untreated hormone in 5 divided daily doses produces an effect equal to that produced in the high concentration of S.L.S. Administration of the hormone as an insoluble copper combination produced an even greater increase in ovarian weight. The results of experiment VI show that S.L.S. by itself has no gonadotropic action, and when administered at a site removed from that at which the gonadotropin was administered is without

augmentation effect. The apparent augmentation produced by S.L.S. would appear to be a result of delayed resorption. Since the addition of S.L.S. to the gonadotropin at pH 7.0 does not produce a precipitate the phenomenon cannot be explained on the basis of insolubility, unless the gonadotropin forms an insoluble complex after injection. The gonadotropin is precipitated by S.L.S. at pH 4.0 and experiments VIII to X are concerned with the administration of the insoluble compound. These experiments show that the gonadotropin is incompletely precipitated by S.L.S. Putnam and Neurath (2) have shown that for certain proteins low concentrations of detergents produce complete precipitation while higher concentrations bring about redispersion. To circumvent this possibility the gonadotropin in experiment X was precipitated fractionally. The precipitate on adding 2 mgm. S.L.S. to 10 mgm. hormone in 6 cc. saline plus .2 cc. 1 per cent acetic acid was removed by centrifugation. One milligram additional S.L.S. produced additional precipitate, which was again removed by centrifugation. Additional S.L.S. produced no precipitate. The ovarian weight of 35 mgm. corresponds to a 45 per cent recovery of potency, so that the biologic activity is equally dispensed between filtrate and precipitate and is fully accounted for. It is apparent that the precipitate is too relatively soluble to effectively delay resorption.

In the rats which had received pituitary gonadotropin in 1 per cent S.L.S. concentration, moderate to severe indurations at the site of injection were observed at autopsy. Single 0.25 cc. injections of S.L.S. in graduated concentrations of 0.25, 0.5 and 1.0 per cent were administered subcutaneously in groups of 3 rats. On autopsy 4 days later slight indurations were observed at the injection site only in those rats which had received the highest dose. An explanation of the mode of action in delaying adsorption may be gained from the following experiment. Six rats were given 1 cc. injections subcutaneously in the inguinal region of a 1 per cent NaCl solution containing 5 mgm. phenolphthalein per 10 cc. Another 6 rats received 1 cc. injections at the same site of a 1 per cent S.L.S. solution containing 0.8 per cent NaCl to bring the ionic strength to the same concentration of the 1 per cent NaCl solution, and 5 mgm. phenolphthalein per 10 cc. One hour after injection the rats were killed. The controls, having received 1 per cent NaCl solution, showed no visible effects at the injection site and there was no evidence of the presence of phenolphthalein on treatment with N/10 NaOH. In contrast the rats which had received the S.L.S. showed a marked edematous area, with a pocket of fluid, which gave a strong test for phenolphthalein on the addition of N/10 NaOH. The action of S.L.S. may therefore be accounted for by a flow of extracellular body fluids to the site of injection thus ultimately delaying the resorption of the hormone.

In the experiments in which insulin was incubated in 5 per cent S.L.S. concentration and then diluted before assay to 0.12 per cent S.L.S. concentration, there is no indication of loss in potency on the basis of the Lacey assay procedure. These results, based on mean fall of blood sugar, were 97 ± 9 , 99 ± 6 and 99 ± 3 per cent respectively (table 3, expts. CS, GH and IJ). The method of Lacey assumes that there is no delayed resorption of the insulin. A comparison of the drop in blood sugar the 5th hour after insulin administration in

the 3 preceding experiments, shows that in each case, the mean blood sugar value was lower for the insulin treated with S.L.S. The differences between the treated and untreated insulins, were respectively 5, 1.5 and 4 mgm. per 100 cc. These results individually were not significant, though taken collectively, they indicate a very slight delay in resorption. Such a slight delay was also produced by 0.1 per cent S.L.S. concentration in the case of the pituitary gonadotropin.

In the experiments in which the assay was performed with an insulin containing 0.5 per cent S.L.S. concentration (table 3, expt. OP, table 4, expt. AB), there is no indication of a delay in resorption, but actually an indication of destruction of activity. In the experiment in which the large dose of insulin was given, the value for the S.L.S. insulin returns to normal the 9th hour while the control is still below normal. The 3rd and 6th hours, the S.L.S. insulin shows a significantly lessened effect. The same result in general was obtained when the assay was performed by the Toronto method, the recovery 82 ± 6 per cent being significantly less. In this case the result the 5th hour was lower for the control than for the insulin containing S.L.S. On increasing the S.L.S. concentration to 1 per cent in the assay solution, a marked decrease in activity is

TABLE 4

Effect in rabbits of 1 U. per K of insulin administered in 0.5 per cent S.L.S. solution

	FASTING	3 HRS.	6 HRS.	9 HRS.	DOSE
A U. 4 Iletin	(103)	43	69	87	1 U/K
B U. 4 Iletin in 0.5% S.L.S. pH 3.0 . . .	(103)	60	93	102	1 U/K
B-A (sugar values \pm Std. Dev. Mean)		17 ± 5	24 ± 8	15	

indicated, the mean fall in blood sugar being 45 ± 5 per cent of the control value in experiment VW and 65 ± 5 per cent in experiment XY. In these experiments the mean blood sugar value the 5th hour is the same as that of the control series. Since there would be on the basis of the experiments with the gonadotropins some delay of resorption of insulin in 1 per cent S.L.S. solution, the destruction of the insulin would actually be even more marked than the results of the assay, which do not account for the augmentation produced by delayed resorption, would indicate.

DISCUSSION. The studies of Miller and Anderson (8) indicate that under the conditions insulin was subjected to S.L.S. in our experiments a high degree of denaturation of the insulin was inevitable. Miller and Anderson did not subject their product to bioassay nor study the sedimentation rate after separation of the insulin from the detergent to determine whether the denaturation was reversible. Rothan, Chow, Greep and van Dyke (9) found that the hormonal activity of insulin is rather insensitive to even high degrees of denaturation. Their observation is confirmed by the results of our experiments, in which the denatured insulin was injected in a low concentration of S.L.S. (0.12 per cent). The decrease in activity observed when the insulin was injected in 0.5 per cent and 1.0 per cent concentration can be accounted for only on the assumption that

the denaturation of insulin by S.L.S. is reversible, and that when the denatured form is injected (in the presence of sufficient S.L.S.) it is destroyed in vivo before the body fluids sufficiently dilute the S.L.S. concentration so that it can revert to its native or more associated state. While this observation, that the denatured hormone is more readily destroyed in vivo than the native form, has not been reported before, the fact is not entirely surprising since it is known that denatured proteins are more readily attacked by proteolytic enzymes. They are probably more vulnerable in other respects also, as in the attack on the sulfhydryl groups.

It is of interest to compare our results on the denaturation of the protein hormones with those reported by Li (10) for the lactogenic hormone. Li found that the denaturation of the lactogenic hormone by urea was reversible and the biologic activity was not destroyed by the denaturation. This observation is to be contrasted with the results obtained by us for the gonadotropins, which were irreversibly denatured by urea. Li found the biologic activity of the lactogenic hormone was decreased when assayed in the presence of the detergent, so that the results resemble those obtained by us with insulin. Li has assumed that inactivation is the result of denaturation, but in view of our results with insulin, in which full activity was recovered when the detergent was sufficiently diluted in the assay, the results reported by Li might be accounted for by an in vivo inactivation of the denatured hormone as we found for insulin.

SUMMARY

The subcutaneous injection of sodium lauryl sulfate produces an edematous area, which may be effectively used in delaying resorption of the gonadotropins, thus producing the well known augmentation of the effect of the sheep pituitary and chorionic gonadotropins. Insulin under these conditions is partially destroyed.

Insulin and the gonadotropins are not inactivated by incubation with sodium lauryl sulfate in vitro. Sodium lauryl sulfate effectively delays the resorption of isotonic solution of phenolphthalein from a subcutaneous injection depot.

The denaturation of insulin by sodium lauryl sulfate is a reversible process, in which the denatured form is inactivated in vivo but not in vitro.

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VITAMIN DEFICIENCY AND OVERDOSAGE AND THE RESISTANCE OF RATS TO LOWERED BAROMETRIC PRESSURES^{1, 2, 3}

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It has been demonstrated that the resistance of laboratory animals to reduced atmospheric pressures is decreased by the administration of thyroid material (Streuli, 1918; Duran, 1920) and increased by surgical (Barach, Eckman and Molomut, 1941) and chemical (Gordon, Goldsmith and Charipper, 1944; Leblond, 1944; Hughes and Astwood, 1944) thyroidectomy. These methods are not without their hazards and it was considered desirable to find other means safely to increase the animal's resistance to anoxia.

The literature reviewed by Carpenter and Sharpless (1937) and Drill (1943) contains numerous references to the relation between thyroid function and vitamin intake. The results are controversial and although conclusive proof as to the actual value of the vitamins as antithyrogenic agents is lacking, the evidence is such as to make it seem worth while to ascertain whether an abnormal vitamin intake, deficiency or overdosage, would influence the resistance of rats exposed to lowered barometric pressures. This paper therefore concerns itself specifically with this problem.

EXPERIMENTAL. The investigation was conducted with 200 adult male rats. These fell into 2 categories.

The first consisted of 40 rats of an inbred-hooded Wistar strain divided equally into 5 groups. The control group was carried on the regular stock diet, and the 4 others received supplements of 1 gram of thiamine, 1 gram of calcium pantothenate and 10 grams of ascorbic acid per kgm. of food respectively. After 21 days on this diet, one half of the animals were subjected to the lowered barometric pressures and sacrificed. The remaining animals were continued on the control and experimental rations. The vitamin B₁ dosage was increased to 2 grams/kgm. on the 23rd day, and the calcium pantothenate to 1.5 gram on the 26th day and to 2 grams/kgm. on the 37th day. The vitamin C diet was maintained at 10 grams/kgm. for 36 days, at which time the concentration was doubled and so kept until the 48th day when all animals were tested for resistance to low pressures.

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² A preliminary report has already been published (Charipper, Goldsmith and Gordon, 1944).

³ Animals and diets used for experiments summarized in table 1 were supplied by the Merck Institute for Therapeutic Research through the courtesy of Drs. Hans Molitor and H. J. Robinson with the assistance of Mr. R. B. Stebbins.

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The rats in the second group were supplied by Carworth Farms and were carried for 2 months on purified diets deficient in either thiamine, riboflavin, pyridoxine or pantothenic acid⁴.

Thiamine series. Twenty-eight animals were maintained on the B₁ deficient synthetic ration, 13 being given 1 mgm. of B₁ by stomach tube 1 to 4 hours prior to exposure in the chamber. Four other sets of 15 rats were fed 1, a restricted amount of the complete purified diet with adequate amounts of all vitamins, so that a weight loss comparable to that suffered by the thiamine deficient rats ensued; 2, the complete purified B₁ diet plus 25 gamma of thiamine daily; 3, the complete purified ration given ad lib, and 4 the ordinary stock diet ad lib.

B₂, pantothenic acid and B₆ series. Three series of 15 rats each were fed the riboflavin, pantothenic acid and pyridoxine deficient diets. Seven of each of these were given supplements of 1 mgm. of B₂, 2 mgm. of calcium pantothenate respectively 2 hours prior to the test and 0.25 mgm. of B₆ 3 hours before exposure to the decreased pressures. In all sets, controls were carried on the complete purified ration, both restricted and the ad lib.

The resistance of these animals to lowered atmospheric pressures was ascertained by the use of a specially constructed illuminated chamber. The pressure was reduced to 190 mm. Hg, (34,000 ft.) over a period of 20 to 40 minutes. This period is not included with the exposure time. The animals were maintained at 34,000 feet and the time of complete respiratory failure of each animal was recorded. Those animals which survived were subjected to a further reduction in pressure. At the conclusion of the experiment representative animals were autopsied.

RESULTS. The data of the deficient and purified ration series are summarized in table 1. Examination of the mean survival time values reveals that the restricted and B₁ deficient animals displayed the greatest resistance, whereas the pyridoxine, riboflavin, and pantothenic acid rats were either as resistant or less so than the controls which had free access to complete diets.

Administration of 1 mgm. of B₁ to the B₁ deficient rats 1 to 3 hours prior to the test decreased their tolerance. Addition of B₂, B₆ and calcium pantothenate supplements to the respective deficient series did not alter the survival times.

Diets high in individual vitamins given to normal animals for periods up to 48 days produced no significant difference in the survival time.

DISCUSSION. Smith, Oster and Toman (1944) observed that cats on limited and thiamine deficient rations were better able to maintain respiration at lower oxygen tensions than well-fed controls. Our data for the rats subjected

⁴ The B₁ deficient diet consisted of casein (Labco), 24 per cent; sucrose, 60 per cent; salt mixture, U. S. P. XI, no. 1, 4 per cent; Crisco, 10 per cent, cod-liver oil, 2 per cent. It was supplemented with 1 gram of inositol, 1 gram of nicotinic acid amide and 10 grams of choline dissolved in 500 cc. of 25 per cent ethyl alcohol, and 200 mgm. of B₂, 100 mgm. of B₆ and 400 mgm. of calcium pantothenate per 10 kgm. of food. The B₂, B₆ and pantothenic acid deficient rations contained 100 mgm. of thiamine per 10 kgm. of the diet.

to B₁ deficient and restricted diets are in general accord with these facts, differing, however, in that our B₁ deficient rats did better than those which were on a complete but restricted diet. That partial inanition in the cat and rat is a factor in itself is inescapable. However, that thiamine does play an important part is emphasized by the reduction in resistance suffered by the thiamine deficient animals given 1 mgm. of thiamine one to several hours before the test. An explanation of this fact might be found in the work of Williams and Kendall

TABLE 1

Action of individual deficiencies of thiamine, riboflavin, pyridoxine, pantothenic acid and a restricted purified diet on the resistance to low barometric pressures

TREATMENT	NO. OF RATS	AVER. FINAL WT.	AVER. SURVIVAL TIME
		gm.	min.
B ₁ defic.....	15	163 (130-204)	61.3(6)* (16-106)
B ₁ defic. + B ₁ prior to exposure.....	13	161 (125-200)	37.3(2) (7-60)
Complete purified + daily suppl. B ₁	15	269 (218-320)	32.3 (12-54)
B ₂ defic.....	8	146 (108-180)	15.7 (1-24)
B ₂ defic. + B ₂ prior to exposure.....	7	152 (125-174)	21.3 (7-29)
Pantothenic defic.....	8	227 (200-265)	24.9 (11-38)
Pantothenic defic. + Ca Pantot. prior to exposure..	7	213 (174-274)	22.6 (4-48)
B ₆ defic.....	8	204 (146-250)	36.1(1) (4-58)
B ₆ defic. + B ₆ prior to exposure.....	7	189 (112-245)	44.5(1) (6-86)
Complete purified restricted.....	30	173 (150-215)	45.9(8) (3-83)
Complete purified ad lib.....	27	268 (200-335)	32.6 (9-82)
Stock diet ad lib.....	15	263 (228-301)	31.9 (19-54)

* The number of animals which survived the 2 hr. exposure to 190 mm. Hg (34,000ft.) are indicated in parentheses. They succumbed when the pressure was reduced to 141-128 mm. Hg (40-42,000 ft.).

(1943) who concluded from clinical studies that thiamine deficiency results in a reduction in the effectiveness of the thyroid in promoting metabolic activity. Should this be true for the rat then a thiamine supplement given to a vitamin deficient animal might be expected to bring about a rise in the metabolic rate. In view of the known relationship between metabolism and resistance to anoxia it is perhaps not surprising that the B₁ deficient animals displayed a decreased tolerance following a single dose of B₁.

King et al. (1945) have presented detailed data to show that partial inanition and a high protein diet decrease tolerance and that pre- and inflight meals high in carbohydrates increase tolerance of the aviator. It should be emphasized that in this work the term "increased tolerance" has been used to denote better survival rather than better performance under anoxic conditions.

SUMMARY

Animals fed limited amounts of a complete synthetic ration and those given a diet deficient in B₁ for 2 months displayed a greater resistance than did rats given B₂, B₆, pantothenic acid deficient diets, and controls which had free access to a complete purified or a stock food diet.

Administration of 1 mgm. of thiamine to the B₁ deficient animals shortly before exposure to the reduced pressure resulted in a decreased tolerance. Similar pre-exposure additions of vitamins B₂, B₆ and calcium pantothenate to the respective deficient diets gave no significant results.

Rats maintained for periods up to 48 days on diets containing supplements of thiamine, ascorbic acid and calcium pantothenate did not differ significantly from stock ration-fed controls in their resistance to reduced atmospheric pressures.

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RESPIRATORY FLOW IN MUSTELUS

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The sharks are convenient for respiratory studies since their skin seems to be more resistant to handling than that of fishes covered with scales or mucous secretion: even when in good health they are docile; their form makes it possible to separate their respiratory intake from the output.

Darbishire (1907) showed, as is now generally accepted, that in the dogfish (*Scyllium canicula* and *Scyllium catulus*) water flows in through the mouth and spiracle and then, as the mouth is closed, out through the gill slits. The present report extends these observations by a consideration of the hydrodynamics and control of respiration.

TECHNIQUE. The observations were all made on *Mustelus californicus* from two to three feet long, caught on set lines, and transferred quickly from the hook to aquaria, without more than a few minutes' exposure to air.¹

The respiratory frequency was counted each day before the fish was disturbed, usually before it had seen the observer, and the temperature of the water was recorded. The fish was then transferred to the respiration chamber.

Respiration chamber. A long wooden trough of appropriate shape and size for the fish was divided by a rubber septum into a "head end" and a "tail end." The shark was threaded through a hole cut in this septum to make a good fit. The head end had a water intake and water level control; the tail end had an overflow the level of which was adjustable. A pair of manometers indicated the difference in level between the water in the two chambers.

The earliest tests on each fish were made under urethane or chloretone anesthesia. After training the fish would remain quiet without anesthetic and all experiments here reported were on fish which had not been anesthetized during the past twenty-four hours.

Respiratory frequency. The respiratory frequency was determined by the temperature (fig. 1); besides this the only variations observed were stoppage, slowing and irregularity. No acceleration was ever observed above (Lutz, 1930) what appeared to be the basic rate in a particular experiment. Thus, the first view of the experimenter, anesthetization, the excitement or exercise of being chased around a long tank for one minute, or the excitement or exercise resulting from an insufficient respiratory flow, produced temporary slowing of respiration. Wide variations in respiratory flow produced by the method of pressure variation were without effect on rate; so also were changes in the pH and oxygen content of the incoming sea water.

¹ A 10 to 30 minute exposure to air at 18° to 23° C., from which the fish apparently recover, is commonly followed in a few days by death or by a long period of relative sluggishness. This may be due to partial desiccation of the gills.

Respiratory flow. The quantity of water flowing through the gills each minute was constant for any fish at constant temperature when the water level in the two compartments was equal (e.g., 395 to 400 cc./min. one day, 348 to 385 cc./min. the next day). The average flow in these circumstances was about half a liter to one liter per minute per kgm. fish varying with temperature (fig. 1) and with uncontrollably small variations in the pressure between the two sides of the diaphragm² (fig. 2).

Small differences in pressure between the two parts of the chamber were accompanied by marked differences in respiratory flow (fig. 1). With the menisci unsteady because of slight movements in the water it was impossible to read the pressure differences precisely, yet an attempt was made to subdivide millimeters

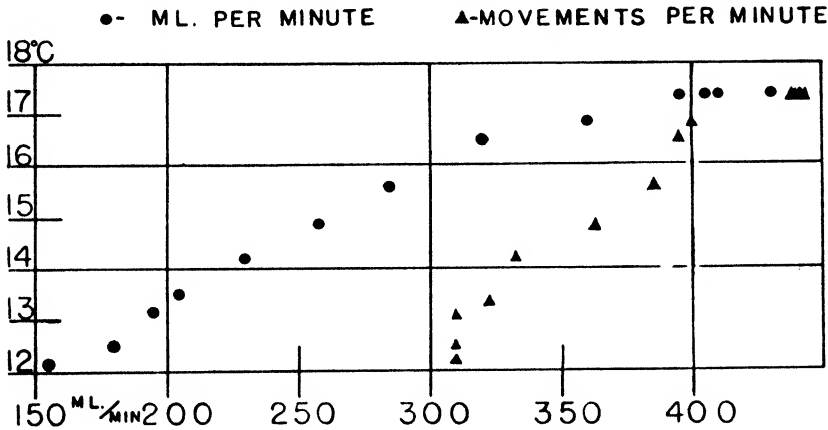


Fig. 1. Influence of temperature on respiration. The data are taken from one experiment on one fish. The measurements are not corrected for the weight of the fish. Note each flow measurement is paired with a rate measurement at the same temperature made simultaneously.

since the total range of pressure was short and the effect of small pressure differences apparently very great.

Examination of figure 2 shows that within the range considered (plus 3 mm. to minus 7 mm.) flow is a nearly linear function of the difference in water level between the head and the rest of the body; and the function is such that every millimeter of lowered pressure at the head diminishes the respiratory flow by about 80 mm. per kgm. fish per minute. Over the whole of this range there is no change in the respiratory frequency. It is probable that the rate of change of flow in the experiments here reported was not as sudden as that in those reported by Lutz who found vagal inhibition of respiration when she changed the rate of flow. When the flow was lowered to one quarter of its natural value (negative pressure of 7 mm.) the fish would wriggle out of its collar and rapidly

² In three anesthetized rays in which the flow was measured by a different and less natural technique the flow was about the same.

resume its normal tranquility in a new position. This wriggling probably corresponded to the generalized muscular activity reported by Lutz when her fish were entirely deprived of gill water. At no time did we observe any increased respiratory effort. It seems likely that in nature anything causing respiratory insufficiency, such as the obstruction of the respiratory passages by seaweed or such as a fish being in a relatively closed region where the water rapidly became unfit for respiration, would yield more satisfactorily to somatic locomotor movements than to increased respiratory effort. That is to say, the fish could get away from the obstruction or the stale water by swimming to

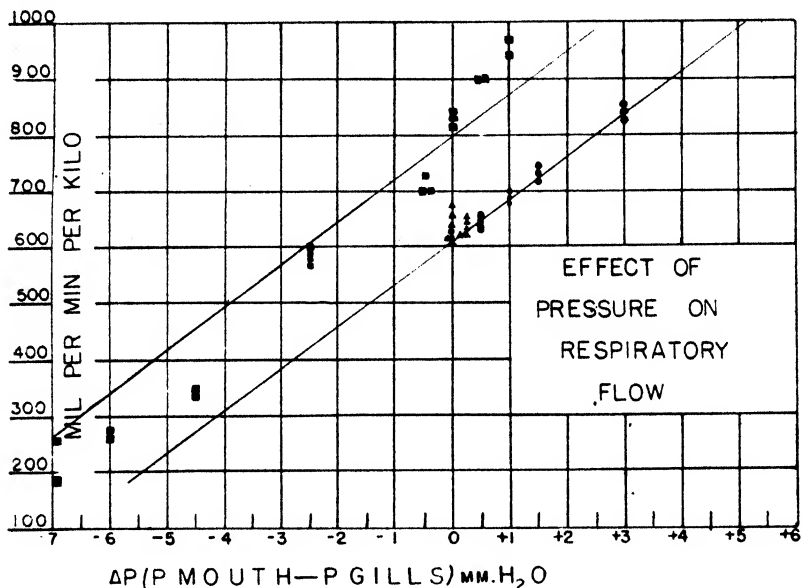


Fig. 2. Effect of pressure on respiratory flow. Dots and squares are from different fish. Triangles are from the same fish as the dots but on a different day. Corrected for weight of fish. The two oblique lines are drawn at a slope to indicate a change of 80 ml./kgm./min. for every 1 mm. change of level, and are only intended for comparison with the data.

another place. This reaction is in contrast to that of mammalia in which respiratory insufficiency gives rise first to increased respiratory depth and rate and only after these have proved of no avail to movements of a more general nature.

Influence of chemical environment. In order to test further the resistance of these fish to respiratory insufficiency, analyses were made of the water entering and leaving the respiratory passages. Two siphons were placed, one in the front chamber and the other in the back chamber with its opening about 1 cm. behind the last gill slits. From these were drawn samples for analyses for oxygen by the Krogh-Winkler (1935) method, for carbon dioxide by the Van Slyke method as modified by Greenberg, Moberg and Allen (1932), and for pH by

colorimetric comparison in a double wedge colorimeter using cresol red and phenol red as indicators.

Repeated analyses showed that the composition of the water was stable during any period in which the respiratory flow was constant and in which no adjustment had been made to the composition of the incoming water. Accordingly the time lag of a half minute or so between the sampling of the inspired and expired waters was negligible. That the samples of water from behind the gills were representative of the water actually leaving the fish and were not diluted by water stirred down from the surface, was shown by the fact that when two immediately successive samples were taken, the oxygen analyses would commonly check within two per cent. Perhaps this merely indicated that the mixing is constant. However, in a few instances samples which in one case had been taken as above into a beaker under oil and which in the other case had been taken directly into the syringe whose needle was within two millimeters of the penultimate gill slit (with traction on the plunger exerted only during the expiratory phases of respiration) were identical. Furthermore, the gills were submerged by at least 3 cm. and no surface currents were visible. The samples for CO_2 and pH analysis were drawn into Winkler bottles, stoppered and stored in the refrigerator. Analyses were completed on the day of the experiment, but tests showed that the composition of the samples was not measurably changed by 24 hours' storage. Studies were made when the composition of the sea water was altered by the addition of CO_2 or HCl and when its oxygen content was changed by bubbling hydrogen, nitrogen or oxygen through it.

Effect of pH. At a natural flow the water entering the tank was found to have pH 8.0 to 8.2 and that leaving the gills pH 7.5 to 7.8. A reduction in respiratory flow resulting from lowered intake pressure produced a lowering of the pH of the expired water. The fish would generally struggle free if the expiratory pH were lowered by this method to less than 7.5. If the pH of the inspired water were slowly lowered by the admixture of water acidified with HCl or CO_2 , the fish would not struggle until the input water was down to pH 6.8 to pH 7.0. In such a case the expired water was always less acid than the inspired water, the most usual value for the expired water being pH 7.2.

In no case did changes in the pH of the inspired water produce a change in respiratory flow.

Effect of oxygen lack. The fish with a natural respiratory flow absorbs oxygen from water containing 5 cc. of O_2 per liter and leaves in the expired water about 1 cc. O_2 per liter giving an oxygen consumption of about 4 cc. per liter or between 1 cc. and 2 cc. O_2 per kgm. of fish. The carbon dioxide production is always a little less, giving a respiratory quotient of about 0.85 to 0.95.

When the oxygen tension in the inspired water which normally was about 130 mm. Hg was artificially reduced to about 50 mm. Hg the characteristic struggling would begin.

These general statements about the chemical aspects of respiration in elasmobranch fishes seem warranted by the data available and of interest enough to report. It has not yet been possible to undertake the more extensive blood and

water analyses which should be done to define these matters with proper accuracy.

DISCUSSION. In summary it may be said that diminution of the availability to the gills of the normal respiratory medium (water), diminishing the available oxygen or establishing conditions unfavorable to the elimination of acid metabolites, are without effect on the respiration of these sharks and produce neither acceleration in rate of gill movement nor increase in the volume of the tidal water. When the need for better ventilation becomes paramount, that is at a definitely lowered pH or O_2 tension, bodily movements of a locomotor nature are instituted. The fish appears to seek a better place. It should not be difficult to locate, by appropriate extirpations, the part of the nervous system which institutes these movements under the stimulus of respiratory deficit.

A comparison of this outline of the shark's respiratory mechanism with that of a man raises interesting points. In general, the vital functions of man are carried out by smooth muscle under the control of the autonomic nervous system. Respiration, by contrast, is carried out through anterior primary divisions of spinal nerves by the segmental intercostal muscles and by the diaphragm. This is a completely "somatic" arrangement and differs from the "visceral" organization of the cardiovascular, digestive, excretory, and other vital systems. This arrangement in the mammal might perhaps arise from the fact that a fish, deprived of effective respiration whether by blockage of the gills by weeds or mud or whether by being in a region of poorly aerated water, can never improve the situation except by locomotion.

Another interesting point of comparison is that the partial pressure (50 mm. Hg) of oxygen at which the fish starts bodily movements is about that at which anoxic effects become important in man. It should be noted that one of the striking signs of acute dangerous anoxia in man is jactitation. These generalized jerking movements of the body are worse than useless in a man whose metabolic needs have dangerously exceeded his oxygen supply but would be effective if as in the fish they helped to translocate him to a place where oxygen was more readily available.

SUMMARY

The flow of respiratory water in the dogfish was about half a liter per minute per kilogram. A nearly linear relation was observed between the quantity of water pumped and the hydrostatic pressure at the front of the head if the pressure outside the gill slits be taken as zero.

No respiratory mechanisms were observed which would tend to compensate for an excess or deficit of respiratory water resulting from such pressure changes. When the respiratory deficit became too great, the fish attempted to swim away.

The respiratory frequency at constant temperature is independent of the respiratory flow, and may be slowed by excitement or anesthetic.

Reduction of oxygen tension or marked reduction of pH in the inspired water produced a swimming reaction.

The respiration of elasmobranch fish is compared with that of man in a short teleological discussion.

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THE NUMBER AND RELATIVE CONCENTRATIONS OF PROTEIN CONSTITUENTS OF CANINE PANCREATIC JUICE AS DETERMINED BY ELECTROPHORESIS

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Reports in the literature have indicated that pancreatic juice contains several proteins. Fractionation of human pancreatic juice with ammonium sulphate by Glaessner (1), Ellinger and Cohn (2) and Wohlgemuth (3) showed the presence of albumin and globulin. De Zilva (4) reported that a part of the protein in canine pancreatic juice is a nucleoprotein, but Wechsler (5), after studying the same fraction, claimed that it was neither a nucleoprotein nor a glucoprotein, although it had a low nitrogen content (13.2 per cent). Langstroth, McRae and Komarov (6) determined the ϵc values (ϵ = extinction coefficient, c = concentration), from absorption spectrum measurements, and the protein nitrogen on various samples of pancreatic juice. Their data showed that the ratio of the ϵc values to protein nitrogen values was a constant, even though the total protein content varied over a wide range. They suggested that this finding indicated that either *a*, only one type of absorbing protein was present, or *b*, if several types were present, they were always present in constant proportions. Babkin (7) has recently reviewed the literature showing that the three best known enzymes of pancreatic juice, trypsin, lipase and amylase, are secreted in parallel concentration. He has suggested that the data of Langstroth, McRae and Komarov might be explained in two different ways: *a*, that, if only one type of absorbing protein is present, it must serve as a carrier for all the enzymatically active prosthetic groups, or *b*, that, if several absorbing proteins are present, they must be secreted in constant proportion whether they are enzymatically active or not.

Electrophoretic investigation of pancreatic juice provides a means of determining which of the above interpretations is correct. First, the number, relative concentrations and mobilities of the electrophoretic components of the juice may be determined, and, second, the various components may be isolated and their enzymatic activity measured. This report deals with experiments designed to accomplish the first step in the analysis.

EXPERIMENTAL. Materials and methods. Pancreatic juice was collected from six unanesthetized dogs through a temporary glass cannula inserted into the main pancreatic duct via a permanent duodenal fistula. The duodenal fistula had been prepared at a previous aseptic operation and was so placed as to be opposite the entrance of the duct into the duodenum. It was fitted with a relatively large fistula tube, the minimum internal diameter of which was five-eighths of an inch. A tubulated gastric fistula was prepared at the

same time. This served, during the collection of pancreatic juice, for the passage of a rubber drain tube through which the gastric contents were drained to the outside. In this way uncontrolled stimulation of the pancreas by the presence of gastric juice in the duodenum was avoided.

These methods have been described in greater detail in previous communications by one of us (J. E. T.) with others (8-11).

Various stimuli were used to promote secretion of pancreatic juice. Acid or neutral peptone solutions, soap solution or N/10 hydrochloric acid were injected intermittently in measured amounts into the upper intestine or secretin was given intravenously by continuous injection. Each of these stimuli produces a distinctive type of secretion having a characteristic range of specific gravity and total nitrogen content (10), except that the secretion formed in response to hydrochloric acid resembles that obtained with secretin.

For the purpose of this study, all cannulas, tubing and glass-ware with which the pancreatic juice came in contact were boiled immediately before beginning the collection to eliminate traces of active trypsin and to decrease bacterial contamination. The juice was then collected into an iced flask and kept at 4°C. or below throughout the study. Juice handled in this way contained no detectable active trypsin at any time during the analysis. This is an essential consideration since pancreatic juice collected without these precautions soon undergoes self-digestion as indicated by the appearance of tyrosine crystals and an increase in nonprotein nitrogen. Exposure of the pancreatic juice to the voltage required for electrophoretic fractionation over a period of four hours caused no reduction in the tryptic activity measured after activation with enterokinase.

Electrophoretic analyses were carried out in the apparatus described by Tiselius (12), equipped with the Toepler schlieren optical arrangement as modified by Longsworth (13). A single section cell of 11.0 ml. capacity (14) was used. The fraction of the total protein contributed by each of the electrophoretically separable components was calculated from the descending pattern as the ratio of each component area to the total area (exclusive of the ϵ -boundary). The method suggested by Tiselius and Kabat (15) was used for allocating the area of each peak. Electrophoresis was allowed to proceed at approximately 5.3 volts for 190 to 240 minutes depending on the nitrogen concentration of the sample.

The samples of pancreatic juice were fractionated without dilution in most cases. The juice obtained with neutral peptone stimulus was diluted with an equal volume of buffer because it was obtained in relatively small amounts and had a high nitrogen content. All samples were studied in a buffer containing 0.025 *N* sodium bicarbonate and 0.1 *N* sodium chloride at pH 8.2. In some cases electrophoresis was carried out on duplicate samples of juice in a buffer consisting of 0.1 *N* sodium diethylbarbiturate and 0.02 *N* diethylbarbituric acid at pH 8.6 (14, 16). The samples were dialyzed in eighteen thirty-seconds inch cellophane tubing for 18 hours against 1000 ml. of buffer and for 24 hours against 2000 ml. of the buffer. The buffer used for the final dialysis was then

used to fill the electrode vessels of the apparatus. As a routine procedure all samples were packed in ice and centrifuged for 20 min. at 2200 r.p.m.

RESULTS. Data are presented on the number, relative concentrations, and mobilities of the electrophoretic components of canine pancreatic juice as they are influenced by *a*, the buffer used; *b*, the stimulus used to promote secretion, and *c*, the dog from which the juice was obtained. Since some of the experiments can be used to illustrate one or more of the points under consideration,

TABLE 1
Effect of sodium bicarbonate and sodium diethylbarbiturate buffers

	MOBILITIES* (CM. ² /VOLT/SEC. × 10 ³)							PER CENT TOTAL PROTEIN*						
	Components†													
	A	B	C	D	E	F	G	A	B	C	D	E	F	G
Bicarbonate (10 expts.) Times component appeared.....	4	10	10		10	3	10	4	10	10		10	3	10
Mean.....	5.62	4.09	2.64		1.60	1.15	0.63	6.1	37.6	13.0		27.1	12.8	16.1
Standard deviation	0.115	0.153	0.160		0.082	0.062	0.096	3.99	4.34	3.05		6.07	2.05	4.34
Barbiturate (10 expts.) Times component appeared.....	4	10	10	10	2	8	10	4	10	10	10	2	8	10
Mean.....	5.17	4.23	2.86	2.11	1.42	1.22	0.40	10.3	32.5	10.0	19.9	15.2	20.1	14.7
Standard deviation	0.344	0.163	0.206	0.087	0.028	0.056	0.207	3.87	4.51	2.04	3.24	3.32	2.77	3.90

* Calculated on the basis of the descending boundaries. Per cent total protein = ratio of component area to total area, exclusive of α -peak, $\times 100$.

† Letters A to G represent electrophoretic components that appeared in one or more fractionations.

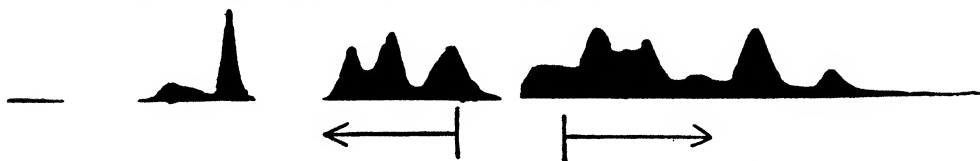


Fig. 1. Electrophoretic patterns of pancreatic juice from dog 1-44 in sodium bicarbonate buffer. Left, ascending pattern; right, descending pattern.

some of the data has been included in more than one of the tables for the purpose of clarification.

a. Sodium bicarbonate and sodium diethylbarbiturate buffers. Comparative data on identical samples of pancreatic juice which were analyzed in sodium bicarbonate buffer at pH 8.2 and sodium diethylbarbiturate buffer at 8.6 are given in table 1. The table presents a summary of results obtained on 10 samples from six dogs. Soap was employed as the stimulus in six cases while each of the other four stimuli was used once. An electrophoretic diagram for pancreatic juice in bicarbonate buffer is shown in figure 1.

Three components, arbitrarily labeled B, C and G, appeared in all the samples of juice studied in both buffers. Component D, which is always present when barbiturate buffer is used, never appears in bicarbonate buffer, whereas component E, which is always present in bicarbonate buffer, appears only twice in the ten experiments in barbiturate buffer. Component F accounts for approximately 20 per cent of the total protein in most fractionations in the barbiturate buffer but is rarely present in fractionations in bicarbonate buffer. The occasional appearance of component F in bicarbonate buffer is not correlated with either the stimulus or dog used. From the data in table 3, where figures are presented on repeated experiments on the same dog, it can be seen that the fastest moving component, A, appears in three of the four experiments with dog 1-44, in all three experiments with dog 2-40, and in only one of the four experiments with dog 2-44. This suggests that the presence of component A is a characteristic of the individual dog rather than of the buffer, the stimulus, or the nitrogen content.

Since identical samples were fractionated in the two buffers, the fact that there is generally an additional boundary in the barbiturate buffer is significant. It appears that a more complete separation of the proteins occurs in the barbiturate buffer at pH 8.6 which results in the appearance of component D. Component F also appears much more frequently than component E. It is suggested that component E may be a mixture which separates on electrophoresis in the barbiturate buffer. The possibility that the proteins have not always completely separated in the bicarbonate buffer is supported by the fact that component F appeared in three cases in this buffer also. Longworth (14) has suggested that this sodium diethylbarbiturate buffer is desirable in fractionation studies on human plasma since it brings about the separation of an additional boundary, the α_1 -globulin, and a more complete separation of the δ -, or ϵ -boundaries from the γ -globulin. The results obtained with the sodium diethylbarbiturate buffer in these studies suggest that this buffer offers some advantages in the fractionation of canine pancreatic juice. However, the greater proportion of the present fractionations were carried out in the bicarbonate buffer since this buffer more nearly resembles the normal medium for the pancreatic juice proteins.

b. *The same stimulus on different dogs.* Soap was used as the stimulus on each of the six dogs studied and the data obtained on these samples of juice in bicarbonate buffer are given in table 2. It can be seen that values obtained on juice from the same dog at different times indicate that there is little, if any, variation in the composition of the juice from the same dog from day to day. However, there is some difference in the number of fractions from one dog to another, especially with regard to component A. If, as has been suggested above, the portion of the total protein comprising fraction F in the two cases where it appears is considered as a partial splitting of fraction E, there is no marked variation in the relative concentrations of the four constant fractions B, C, E, and G from one dog to another except in dog 1-44. With this dog there is a somewhat larger amount of the slowest moving fraction G, and a

TABLE 2
Effect of soap stimulus on the six dogs

EXPT. NO.	DOG NO.	MOBILITIES* (CM. ² /VOLT/SEC. $\times 10^6$)						PER CENT TOTAL PROTEIN*					
		A	B	C	E	F	G	A	B	C	E	F	G
49	2-40	5.68	4.18	2.96	1.73		0.65	7.4	32.4	8.0	35.8		16.4
51	2-40	5.44	3.99	2.79	1.58		0.59	6.1	32.8	6.1	43.1		11.9
58	1-44	5.39	4.14	2.45	1.60		0.71	11.5	32.1	5.5	22.5		28.4
62	1-44	5.51	3.94	2.78	1.57		0.69	9.5	32.6	7.3	26.6		23.9
66	2-44		4.17	2.84	1.68		0.73		41.5	11.8	33.9		12.7
81	2-44		3.94	2.61	1.64	1.10	0.50		39.3	13.8	19.5	16.1	11.2
97	1-45	5.59	4.15	2.47	1.62	1.14	0.60	2.4	42.5	13.6	17.7	9.9	13.8
99	3-45		3.85	2.43	1.49		0.57		42.7	13.8	30.2		13.2
101	F.F.		4.10	2.52	1.48		0.53		39.4	13.4	30.3		16.9
Mean.....		5.52	4.05	2.65	1.60	1.12	0.62	7.4	37.3	10.4	28.8	13.0	16.5
Standard deviation....		0.114	0.121	0.196	0.081	0.004	0.008	3.46	4.69	3.57	8.20	1.39	5.90

Sodium bicarbonate buffer used throughout.

* Calculated on the basis of the descending boundaries. Per cent total protein = ratio of component area to total area, exclusive of ϵ -peak, $\times 100$.

TABLE 3
Effect of various stimuli on three dogs

EXPT. NO.	STIMULUS	DOG NO.	MOBILITIES* (CM. ² /VOLT/SEC. $\times 10^6$)						PER CENT TOTAL PROTEIN*					
			A	B	C	E	F	G	A	B	C	E	F	G
54	Peptone-HCl	1-44	5.49	3.91	2.70	1.60		0.64	9.1	30.6	7.5	21.8		31.0
62	Soap	1-44	5.51	3.94	2.78	1.57		0.69	9.5	32.6	7.3	26.6		23.9
92	Secretin	1-44		4.36	2.78	1.70		0.79		35.8	16.4	24.8		23.0
94	HCl	1-44	5.59	4.15	2.84	1.61		0.73	9.6	29.7	8.6	34.4		17.7
51	Soap	2-40	5.44	3.99	2.79	1.58		0.59	6.1	32.8	6.1	43.1		11.9
52	Peptone-HCl	2-40	5.71	4.29	2.88	1.82		0.77	4.9	31.3	12.0	39.2		12.6
53	Peptone	2-40	4.67†	3.92	2.78	1.63		0.57	12.1†	26.1	13.5	37.6		10.7
66	Soap	2-44		4.17	2.84	1.68		0.73		41.5	11.8	33.9		12.7
69	Peptone	2-44	5.78	4.03	2.49	1.52		0.61	2.9	37.6	14.0	32.0		13.5
79	Peptone-HCl	2-44		4.22	2.61	1.69	1.22	0.58		34.8	17.0	21.1	12.4	14.7
81	Soap	2-44		3.94	2.61	1.64	1.10	0.50		39.3	13.8	19.5	16.1	11.2
Mean.....			5.46	4.08	2.74	1.64	1.16	0.65	7.7	33.8	11.6	30.4	14.3	16.6
Standard deviation.....			0.367	0.161	0.120	0.081	0.085	0.094	3.20	4.52	3.76	8.04	2.62	6.58

Sodium bicarbonate buffer used throughout.

* Calculated on the basis of the descending boundaries. Per cent total protein = ratio of component area to total area, exclusive of ϵ -peak, $\times 100$.

† A-boundary was not completely separated.

smaller amount of fraction E. Enzyme studies¹ on the pancreatic juice from this dog have shown a very poor correlation between the trypsin content and the specific gravity of the juice. This is at variance with the results obtained with other dogs (17). Whether this bears any relation to the electrophoretic patterns cannot be stated at this time.

c. *Different stimuli on the same dog.* The effect of using various stimuli for the production of the juice was investigated in three of the dogs. The electrophoretic analyses of these juices in bicarbonate buffer are presented in table 3. These data indicate that although the protein content of the pancreatic juice varies widely, depending on the stimulus used (0.56 gram protein/100 ml. for the secretin to about 6.3 grams protein/100 ml. for peptone juice), the number and the relative concentrations of the fractions remain fairly constant regardless of the stimulus.

DISCUSSION. Pancreatic juice does not appear to be as consistent as normal plasma with regard to the number of its constituents. From four to six components have been found, depending on the experimental conditions. Although the mobilities of certain of the components of pancreatic juice (B, C, D, and F) are identical with those of some plasma components, there is, as yet, no evidence to justify considering that these are therefore the same proteins. The average values which we have obtained for the mobilities in three canine plasmas, in sodium diethylbarbiturate buffer, are as follows: albumin, 6.15; α_1 -globulin, 5.22; α_2 -globulin, 4.00; β -globulin, 2.88; fibrinogen, 2.15; and γ -globulin, 1.21. Only four of the pancreatic juices fractionated in barbiturate buffer contained the fast moving A component, and it is somewhat surprising that the mobilities are all lower than the values obtained for the A component in the bicarbonate buffer at a somewhat lower pH. The mobility for fraction A in the barbiturate buffer definitely resembles that of α_1 -globulin, while that obtained in the bicarbonate buffer lies between the values for albumin and α_1 -globulin. Furthermore, component A accounts for only 10 per cent or less of the total protein. Dole (18) has conducted the electrophoretic analysis of fifteen normal human plasmas in the same barbiturate buffer used in this study. He reported a mobility for albumin of 5.94 ± 0.267 cm.²/volt/sec. $\times 10^{-5}$, and the mobility given above for albumin in dog plasma falls within this range. No component of canine pancreatic juice was found to have as high a mobility in this buffer. Electrophoretic analysis of pancreatic juice, therefore, suggests that it contains no albumin. This does not agree with the chemical fractionation of Glaessner (1) who reported 62 per cent albumin and 38 per cent globulin. Dole (18) has shown that a part of the plasma globulin, as measured by electrophoresis, is not precipitated by sodium sulphate and thus is measured as part of the albumin in the fractionation by Howe's (19) method. This fact is probably the explanation for the high albumin values reported for pancreatic juice after ammonium sulphate precipitation. It should be mentioned that, although there appears to be a considerable amount of protein in fraction D, in the barbiturate buffer, with a mobility the same as that of plasma fibrinogen,

¹ The enzyme studies have been carried out by Dr. M. H. F. Friedman of the Department of Physiology.

the addition of thrombin to fresh pancreatic juice does not produce a fibrin clot. The investigation, now under way, on the distribution of the enzymes among the various components of pancreatic juice may throw some light on the nature of the proteins present.

Hesslevik (20) reports that an examination of a variety of body fluids (urine, pleural effusion, aqueous humor, and pericardial, cerebrospinal, ascites and hydrocele fluids) reveals the presence of electrophoretically well-defined protein components, but that only three and sometimes two distinct boundaries are regularly found. It is interesting that pancreatic juice with a protein concentration as low as 0.56 gram/100 ml. reveals four, and at times five, distinct components.

The fairly constant values which we have obtained for the relative concentrations of the various fractions in samples of pancreatic juice, with widely varying protein content, from the same dog are good supporting evidence for the findings of Langstroth, McRae and Komarov (6). We have not carried out any absorption spectrum measurements so are unable to state whether each of the electrophoretically separable components is an absorbing protein.

SUMMARY

Pancreatic juice obtained from dogs under the influence of various stimuli has been subjected to electrophoretic fractionation. In sodium bicarbonate buffer at pH 8.2 the juice separates into four or five components depending on the dog under observation, but independent of the stimulus used or the protein concentration of the juice. In sodium diethylbarbiturate buffer at pH 8.6 an additional boundary generally appears. The relative proportions of the several components tend to remain fairly constant.

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THE ACTION OF ACETYLCHOLINE AND EPINEPHRINE ON THE TURTLE VENTRICLE

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In 1934, in a study of the action of certain drugs upon the refractory period and other fundamental properties of heart muscle, Wedd reported that for the turtle heart acetylcholine caused decreased height of contraction and shortening of both the refractory period and the duration of systole, and that the effect was similar on auricle and ventricle. In recent years, Garrey and his collaborators have studied extensively the action of acetylcholine on the turtle heart. They have stressed its so-called inhibitory action. Garrey and Chastain (1937) concluded that chelonian ventricular muscle is insensitive to the inhibitory effect of acetylcholine, and also that inhibitory effects depend upon innervation by inhibitory nerves.

Because in the earlier work, the refractory period was determined by a faulty method, which included conduction, the action of acetylcholine on the turtle heart has been reinvestigated as part of further study of the direct action of drugs on cardiac muscle. It was found by Blair, Wedd, and Young (1941) that when both measurements were made at the same region on a strip of muscle, the direct determination of the absolute refractory period and the Q-T interval of the electrogram gave practically identical values. The theoretical considerations and details of the methods used in recent drug studies have been reported by Wedd, Blair and Dwyer (1941).

The effect of acetylcholine on contractility was observed on ventricular strips suspended in a bath whose volume was about 20 ml. One end was attached to a lever and the beat recorded on a smoked drum. The tissue was stimulated rhythmically by condenser discharges which passed through the strip. Most strips were driven at a rate of 13 per minute; a few observations were made with a rate of 24. In this series, 15 turtle hearts were used, and generally two strips were obtained from each ventricle. Tissues were observed at varying intervals after removal from the animal, from fresh to 120 hours. No relation between such times and the response to acetylcholine was found. Acetylcholine solutions were always freshly prepared from crystals of acetylcholine bromide. Since the drug was usually effective only in concentration far above that required to produce the usual inhibitory effects, most observations were made with concentrations varying from 1:40,000 to 1:10,000. The so-called inhibitory effect was observed but once and that was in a freshly prepared strip. And only once was there no response to a concentration of 1:10,000; that strip had high initial tone and the beat was exceptionally small. The customary response was a slight to marked decrease in amplitude of beat and a

decrease in diastolic length (fig. 1). The effect appeared promptly and at times began to pass off while acetylcholine was still present in the bath. When the acetylcholine solution was removed and the strip was again bathed with Ringer solution, beat amplitude usually recovered promptly. This action of acetylcholine was entirely prevented by atropine when a sufficient quantity of the latter had been first added to the bath. No attempt was made to determine the exact quantity of atropine that would prevent an acetylcholine response, but some observations suggested that approximately an equal weight was required. In one experiment 1.3 mgm. of atropine did not offset 2 mgm. of acetylcholine. However, 2.6 mgm. of atropine in the bath always prevented a response to 2 or 2.5 mgm. of acetylcholine. A few observations were made on the effect of carbaminoyl choline, Merck's "Doryl" being used. The effect of 2 mgm. of that drug was quite similar to that produced by the same quantity

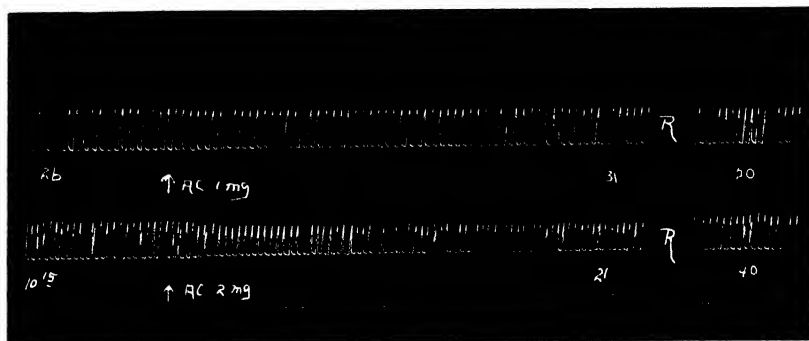


Fig. 1. Illustrating direct action of acetylcholine on a driven ventricular strip. Concentrations, 1:20,000 (upper) and 1:10,000 (lower). The drug was introduced as indicated by the arrows. It was removed and fresh Ringer's solution restored at *R*. The experiment started at 9:26 and the numbers below show the times in minutes thereafter.

of acetylcholine, and its action was likewise prevented by the presence of atropine in the bath.

The effect of acetylcholine on the refractory period was studied by following changes in the Q-T interval of the electrogram. With the method employed, the Q-T interval is the time between depolarization and repolarization of the membrane at a particular region of the strip. The results are given in table 1. With concentrations of 1:100,000 and greater, slight but definite shortening of Q-T occurred in all preparations but one, and in that the initial Q-T interval had already been greatly shortened by the increased rate of beating. The shortening, which was of the order of 5 to 12 per cent, appeared promptly, reached its maximum in from one-half to three minutes, and the reaction was over soon after the tissue was again flushed with Ringer solution. The degree and the timing of the effect are in accord with other evidence that the action of acetylcholine is a surface one. It will be seen that while higher concentrations usually produced greater shortening, the effect was by no means directly proportional to the increase in concentration.

In the same strips in which the refractory period was studied fiber conduction time was also recorded. Two receiving electrodes were placed on the tissue and each paired with an indifferent electrode which was placed on the moist filter paper about 2 cm. from the strip. The Q-Q interval is the conduction time for the length of muscle between the receiving electrodes, approximately 5 to 8 mm. The Q wave was often bound with the deflection caused by the shock, and in general Q-Q was a less accurate and less satisfactory measurement than Q-T. However, from the figures given in table 1, it seems justifiable to

TABLE 1

Effect of acetylcholine on refractory period and conduction

EXPERIMENT	RATE	CONCENTRATION × 1000	Q-T		Q-Q	
			Before	After	Before	After
			sec.	sec.	sec.	sec.
A	24	1:1000	1.14	1.20	0.18	0.20
	24	1:100	1.20	1.14	0.22	0.24
	24	1:100	1.64	1.56	0.28	0.24
	24	1:10	1.64	1.52	0.24	0.24
B	13	1:100	1.56	1.48	0.12	0.12
	13	1:10	1.64	1.52	0.12	0.12
C	13	1:100	1.44	1.34	0.20	0.20
	13	1:10	1.44	1.26	0.22	0.24
D	13	1:50	1.46	1.28	0.26	0.28
	13	1:10	1.46	1.36	0.32	0.30
E	13	1:10	1.48	1.34	X	X
F	13	1:10	1.20	1.10	X	X
	24	1:10	0.57	0.57	X	X

Q-T interval, the refractory period. Q-Q interval, conduction time between receiving electrodes. Rate, beats per minute.

conclude that acetylcholine has no appreciable or consistent action on fiber conduction.

These effects of acetylcholine on contractility and refractory period are believed to represent a direct action on heart fibers, independent of inhibitory action. Such action is a weak one and requires concentrations that may be considered non-physiological. It is not, however, a toxic effect for recovery is prompt and usually complete, and the same muscle strip has been subjected to such dosage on three successive days, always with response and recovery.

Epinephrine on ventricular strips. Hiatt and Garrey (1943) studied the actions of epinephrine on strips of turtle ventricle and found little response, only a slight increase in amplitude without much rate increase, and that it was difficult to repeat the effect in the same preparation. It was suggested that this

response, unlike that of turtle and frog auricles and the frog ventricle, resulted from absence of sympathetic nerves. The slight effect seen was attributed to a direct action on the cell. We have made observations with epinephrine similar to those described with acetylcholine, and they are, in general, in agreement with those of Hiatt and Garrey. Even with concentrations of 1:10,000 the results were strikingly irregular. The mechanically recorded beat at times increased, often only slightly. In some strips the beat decreased, and when there had been a positive inotropic effect, the beat almost always declined when the drug was repeated. No relation was found between the effect and the time the tissue had been out of the animal.

The effect on the electrical responses of strips was likewise very irregular, making it difficult to say that epinephrine has any definite influence on refractory period or conduction. This behavior is in decided contrast to the acetylcholine effect, which while it was never marked, always occurred and in the same direction. It is of interest to note in this connection that epinephrine in the concentrations herein used has no effect on frog skeletal muscle.

SUMMARY

The effect of acetylcholine upon rhythmically stimulated strips of turtle ventricle, presumably free from nerve endings, is to decrease contraction height and diastolic length, and to shorten slightly the refractory period. This action, which requires much higher concentrations than those needed to produce the so-called inhibitory effects, is offset by atropine and is believed to represent a direct action on myocardial fibers. The response to carbaminoylcholine is similar to that of acetylcholine.

The action of epinephrine on such ventricular strips is irregular and inconstant. Contraction may be increased or decreased. There appeared to be no definite effect on refractory period or fiber conduction.

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THE EFFECT OF SODIUM AND CHLORIDE SALTS IN PREVENTING THE SHOCKLIKE STATE FOLLOWING VENOUS OCCLUSION OF A LIMB IN THE DOG¹

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A method of producing shock in dogs by occlusion with lampblack thrombi of the leg veins has been described (1), a procedure leading to a shocklike state followed by death in most untreated animals (2). An invariably fatal result is produced when the veins in both hindlimbs are occluded by this means (2, 3). In single limb venous occlusion some disparity in the fatality rate occurred in different series of animals. Thus, fatal shock was produced in 21 of 23 animals in one series (4), but in a later one 22 of 28 succumbed (2). Various attempts have been made to improve the survival rate of animals with venous occlusion (5, 6, 7). It was found that isotonic saline solution in one series insured survival of 12 out of 12 animals (4). In a second series the results were not so good, although still definite, viz., 13 out of 28 animals survived (4). Isotonic glucose solution in the initial series was not as effective as saline, only 7 out of 12 animals surviving (4).

It was therefore felt that a third independent series was desirable. Furthermore, the partially beneficial action of saline raised two questions: *a*, was it possible to improve upon its action by balancing NaCl with the other ions of body fluids, and *b*, to the extent to which saline therapy substituted for the loss of fluid from the bloodstream, was its action due to specific ion effects or was, as the early beneficial results obtained with glucose seemed to suggest, any innocuous fluid acceptable for replacing the fluid lost into the leg.

To answer these questions, several solutions were used in the present study, besides NaCl and glucose, viz., Ringer's solution, several sodium salts with relatively innocuous acid radicals and lithium chloride (the only chloride which seemed to hold promise of being sufficiently un toxic for use). The sodium salts tested were lactate, dibasic phosphate and succinate. Untreated animals, saline treated animals, glucose treated animals and animals treated with Ringer's solution were interposed with these series.

METHODS. The Ringer's solution was made up in the usual manner. The salt solutions were made isotonic with respect to the ion tested as indicated in table 1 (i.e., not quite isotonic in the case of polybasic acids among the sodium salts). The number, amount and timing of injections are summarized in table 1. All solutions were injected intravenously as described previously (4). Lactate, phosphate, succinate and lithium chloride were given in smaller total

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This work was done under the auspices of the Shock Committee of the Michael Reese Hospital and supported by the Michael Reese Research Foundation.

quantities and more frequently than the Ringer's, glucose and saline solutions, to minimize toxic actions. All solutions were given over the first few hours after completing the operation. Succinate on occasion was given over a longer period. Anesthesia and the surgical procedure used in occluding the veins were the same as those previously employed (1).

RESULTS. The number and percentage of survivals obtained in each group are shown in table 1. The critical survival time was 24 hours; all animals surviving beyond this time were considered survivals.

In the untreated group 7 out of 28 animals survived, compared to 2 out of 23 in our first series and 6 out of 28 in our second series,² a fair agreement.

In the saline treated group, 11 out of 19 animals or 58 per cent survived, compared to 12 out of 12 in our first series and 13 out of 28 or 46 per cent in our sec-

TABLE 1
Summary of results

THERAPEUTIC AGENT USED AND MODE OF THERAPY				WEIGHT OF ANIMALS IN GROUP		NUMBER OF ANIMALS IN GROUP	NUMBER AND PER CENT SURVIVALS	
Agent and concentration (gms./L)	Total amount injected cc.	Number of injections	Duration of therapy hours*	Range lbs.	Average lbs.		Number	Per cent
1. None.....				9-28	16	28	7	25.0
2. Ringer's (mammalian).....	410-810	3-4	1.75-3.50	11.5-29	18	12	11	91.7
3. NaCl (9.0).....	370-800	3-5	2.00-4.50	9-49	16	19	11	57.9
4. Na-lactate (17.24)...	450-780	3-6	2.00-4.25	14-25	20	10	5	50.0
5. Na ₂ -phosphate (19.0)	280-495	5-8	2.00-4.25	14-22	17	10	5	50.0
6. Na-succinate (12.74)...	300-515	5-17	1.75-3.75	11-19	13	14	1	7.1
7. LiCl (6.5).....	200-675	3-8	2.00-4.50	10-25	17	8	0	0.0
8. Glucose (55.2).....	370-730	3-5	2.00-4.00	8-24	14	12	3	25.0

* Zero time being the end of the operation.

ond series.³ In each of the three series, saline solution caused a definite increase in the survival rate over that in untreated animals. The difference in survival between saline treated and untreated animals is statistically significant in the present series.

Ringer's solution was found to be more effective than saline solution, 11 out of 12 animals surviving.

Among the sodium salts tested, lactate and dibasic phosphate proved to be almost as effective as NaCl solution, 5 out of 10 animals surviving in each case. Surprisingly enough, only 1 survival among 14 animals was seen in the sodium succinate group, although great care was taken to avoid toxic levels by giving this solution in very frequent small amounts. The complete lack of effectiveness of succinate early in venous occlusion shock contrasts with its efficacy when given late in developed hemorrhagic shock when acidosis is prominent (8).

Glucose in the present series turned out to have no demonstrable beneficial

² Twenty-one of the animals used in the second series were also used in the present series.

³ Fifteen of the animals used in the second series were also used in the present series.

effect, as the survival rate in this series (3 out of 12 animals) was no better than in the untreated animals in this series (7 out of 28 animals).

None of the lithium chloride animals survived. In spite of the low dosage, toxic symptoms such as retching and vomiting were frequently seen after injection in these animals, although such symptoms were not observed in healthy animals to whom larger doses of lithium chloride were given.

DISCUSSION. The present series leads to the following conclusions: 1. It brings out the greater effectiveness of Ringer's solution as compared to NaCl solution. Apparently, the dilution of the Ca- and K- ions resulting from the administration of saline in large quantities is serious enough to tip the scales against survival in a number of animals.

2. It confirms our previous conclusions (4) as to the greater effectiveness of saline over glucose solution.

3. The results obtained with sodium lactate and phosphate, which were about as effective as sodium chloride, suggest that depletion of the chloride ion can be withstood by these animals to about the same degree to which they can withstand depletion of calcium and potassium.⁴

The discrepancy found in saline, glucose and untreated animals in different series has been discussed previously (2). Even though the attempt was made to keep conditions of the operation and of therapy alike, sufficient differences in series performed at different times must exist to cause variation in results. Possibly the animals in the present series were in poorer condition or the occlusive effect of the injected lampblack was greater.

SUMMARY

It would appear from our studies that in fluid replacement therapy, the main requirement is replacement of electrolyte solution lost into the limb below the venous occlusion. Of the ions lost, sodium appears to be the most important, but chloride, calcium and potassium ions also appear significant.

It should be remembered that these results apply to the early stages in the development of shock. They cannot be carried over to the later stages when shock is becoming or has become irreversible.

We are indebted to other members of the department for their assistance.

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⁴ A comparison of all surviving with all dead animals suggested certain trends:

1. Smaller animals tend to survive better than the larger ones.
2. Smaller amounts of lampblack injected into the veins tend to raise the chance of survival.
3. Smaller quantities of fluid substitution tend to favor survival in each series tested. Apparently, the quantities of solutions used in this series were often beyond the optimum therapeutic dosage.

CHANGES IN THE BLOOD DURING CHRONIC AND ACUTE DEHYDRATION

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A more complete knowledge of the changes in the blood accompanying dehydration is highly desirable in order to determine the severity of the condition when it occurs in man. Anhydremia as a result of dehydration has been reported many times (Marriott, 1923). The principal methods used in estimating the amount of water lost by the blood have involved the determination of changes in the solids of the blood (Rominger, 1920), in the hemoglobin (Rowntree, 1922), in the erythrocyte count (Darrow and Yannet, 1935), in the specific gravity of the blood (Drew et al., 1940), or more recently in the total volume of blood or plasma (Mellors et al., 1942).

Much of this work has involved a comparison of animals sacrificed at intervals. Because chronic dehydration due to water deprivation occurs frequently in man it was considered pertinent to determine the changes that might occur in an animal during a control period followed by dehydration and recovery. A comparison was made with acute dehydration following hemorrhage or diuresis. A correlation of several methods for measuring anhydremia was also considered desirable.

Methods. Dogs weighing 5 to 10 kgm. were selected for this study. Determinations of hemoglobin, red blood cell count, and specific gravity were made in duplicate for several days under normal conditions and continued throughout dehydration and recovery periods. The hemoglobin was determined by the use of the Cenco-Sheard-Sanford photometer, the red blood cell count using the Neubauer counting chamber, and the specific gravity by the Barbour and Hamilton falling drop method as described by Motley (1941). Samples of blood for analysis were removed during the course of experimental treatment and during recovery. Approximately 10 grams of whole blood were accurately weighed and dried to a constant weight at a temperature of $103 \pm 1^\circ\text{C}$ to ascertain total blood solids. Fats were extracted using purified ether.

Nineteen dogs were used in this study. Six of these dogs were dehydrated under acute conditions consisting of hemorrhage of 9-20 grams of blood per kilogram of body weight in 4 dogs and the injection of 100 cc. of 50 per cent sucrose in 2 dogs. In three of these dogs the acute dehydration was produced after a period of recovery from chronic dehydration and in the other three dogs at the height of the dehydration period. Acute dehydration was carried out under pentobarbital sodium anesthesia. The remainder of the experimental animals were dehydrated by water deprivation and inanition for a period of 7 to 10 days. Because of conservation of fluid in the dog, this period is comparable

to a much shorter period in man. The animals suffered no noticeable discomfort and were docile at all times.

RESULTS. Chronic dehydration by water deprivation over a period of 10 days was accompanied by hemoconcentration of the blood as shown by an increase in hemoglobin, erythrocyte count, and specific gravity. In figure 1 the percentage deviation from the normal condition is shown. There was some fluctuation the first and second day followed by a gradual concentration continuing for about seven days. The actual average hemoglobin content of the blood changed from 15.9 grams per 100 cc. blood to 20.7 grams per 100 cc. blood. The red blood cell count increased from 5,151,000 to 6,702,000. The specific gravity of the blood during this time increased from 1.04528 to 1.05201. Dehy-

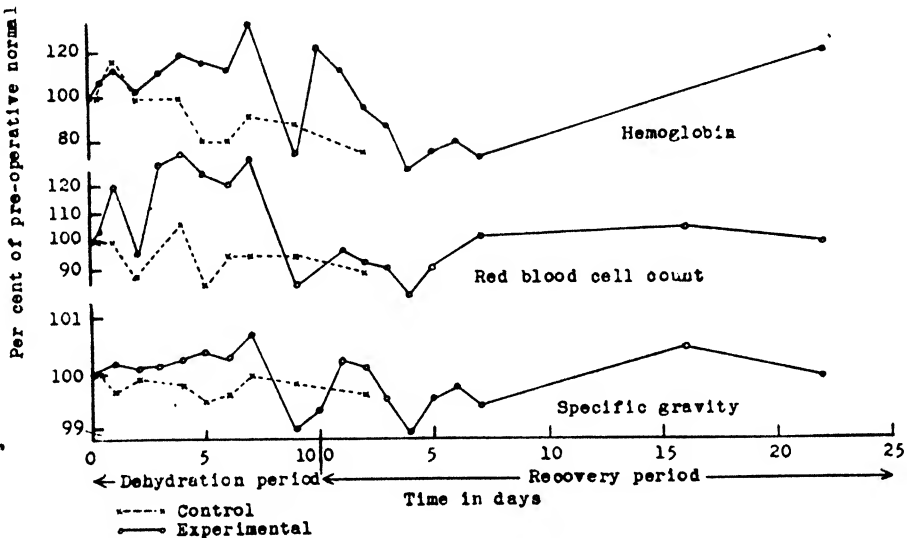


Fig. 1. Changes in the hemoglobin, red blood count, and specific gravity in the blood during dehydration and recovery.

dration for periods longer than seven days was followed by a decline in the per cent of blood constituents to, or slightly below, the normal level. Upon returning the animals to their normal diet there was a sudden hemodilution, and recovery was gradual over a period of 25 days. The water content of the blood obtained from the fifth to seventh day, as determined by desiccation, decreased about 1 per cent, averaging 81.8 per cent in this group of animals under normal conditions and 80.6 per cent at the height of the dehydration. Upon recovery or return to the normal diet the water content of the blood was found to be 84 per cent.

The change in the body weight during the interval of dehydration was most marked during the first 2 days when about 10 per cent of the total weight was lost. By the end of the period about 25 per cent of the original weight had been lost. When the dogs were returned to the normal diet recovery was rapid the

first and second days, during which time the dogs regained to within 10 per cent of their normal weight. This was followed by a period of fluctuation and a gradual return to the normal level in about 25 days.

Acute dehydration following a period of chronic dehydration by water deprivation and inanition showed little effect upon the blood picture. The water content of the fat free blood samples deviated less than 1 per cent from the condition after chronic dehydration. The specific gravity, hemoglobin, and red blood cell count changed but little. Normal dogs dehydrated by acute hemorrhage showed the characteristic hemodilution, with the desiccated blood samples showing 85.5 per cent water as compared to the normal samples of 84 per cent. Fifty per cent sucrose injection was followed by an increase in the blood constituents. Hemoglobin increased from 14.5 grams per 100 cc. to 16.5 grams per 100 cc. of blood. The erythrocyte count deviated from 4,590,000 to 5,210,000 cmm. and the specific gravity was changed from 1.04560 to 1.05140.

DISCUSSION. Dehydration resulted in gradual hemoconcentration, but toward the end of a 10 day period there was a decrease in the blood constituents to a slightly subnormal level. The water content of the blood decreased about 1 per cent and upon recovery the water content of the blood increased above the normal status. With reference to the actual percentage of water in the blood, the changes occurring during dehydration could be interpreted only by comparing the effect of dehydration with the normal condition in the same dog since the individual variation was often greater than the effect of dehydration. These changes indicate that compensation for dehydration involves more than merely loss of water. Rapid hemodilution upon return to the normal diet suggests that there is an actual loss of other blood constituents greater than the analysis of the blood during the period of dehydration would indicate. With a loss of fluid and a somewhat comparable loss of solids from the blood the exact condition of the animal undergoing dehydration is difficult to determine.

Acute hemorrhage accompanying chronic dehydration gives a picture of relative stability of the circulatory medium that also makes it difficult to determine the true state of the animal in question. Acute hemorrhage in the normal dog or dehydration by hypertonic sucrose shows rapid changes characteristic of conditions such as secondary shock. These results indicate that the present methods of determining blood volume based upon hemoglobin, specific gravity, or other blood constituents, although known to be relatively accurate indices in clinical syndromes involving sudden water loss in a normal individual, may not be satisfactory in conditions of water loss produced by prolonged dehydration. This investigation suggests that a relatively normal equilibrium in the vascular system is maintained only by proportionate decreases in total quantities of the formed elements and other constituents concomitant with the decrease in blood volume.

SUMMARY

Prolonged dehydration produced by inanition and water deprivation and acute dehydration induced by hemorrhage or by the injection of hypertonic sucrose solutions have been studied in 19 dogs.

In prolonged dehydration dogs lost 25 per cent of the body weight in 10 days and recovered gradually over a period of 25 days. During prolonged dehydration the water content of the blood decreased 1 per cent. During recovery the water content increased to or above the normal.

During prolonged dehydration the hemoglobin, erythrocyte count, and specific gravity rose slightly during the first few days and then decreased to below the normal level of the animal. Upon recovery these constituents of the blood decreased markedly but gradually returned to the normal values.

Acute dehydration under anesthesia produced by hemorrhage or the injection of hypertonic sucrose solutions caused sudden changes in the water content of the blood and the constituents of the blood in normal dogs but had little effect in dogs which had been without water for several days.

It is suggested that the condition of the animal prior to the removal of blood by hemorrhage or other means in acute conditions is an important factor in determining the changes in the blood picture accompanying this loss of blood.

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These differences in weight gain were much greater in the low protein groups. The control low-fat high-protein and high-fat high-protein groups gained 14 and 44 grams more than their corresponding exposed groups respectively, but these differences were not significant statistically, while the corresponding differences in low-protein groups were very significant, being 55 and 83 grams for the low and high fat diets respectively.

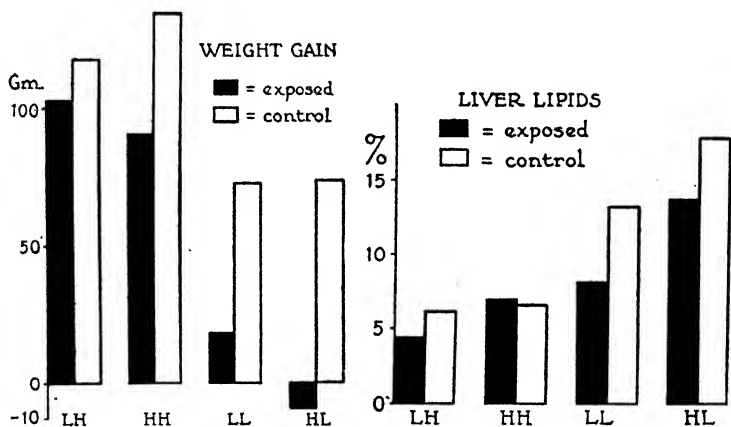


Fig. 2. LH—low-fat high-protein diet; HH—high-fat high-protein diet; LL—low-fat low-protein diet; HL—high-fat low-protein diet.

Fig. 5. LH—low-fat high-protein diet; HH—high-fat high-protein diet; LL—low-fat low-protein diet; HL—high-fat low-protein diet.

TABLE 2
Growth

DIETS	EXPOSED		CONTROL	
	No. of rats	Gain	No. of rats	Gain
		gm.		gm.
Low-fat high-protein.....	10	103 \pm 20	10	117 \pm 16
High-fat high-protein.....	10	91 \pm 21	7	135 \pm 31
Low-fat low-protein.....	10	18 \pm 18	8	73 \pm 18
High-fat low-protein.....	9	-10 \pm 27	9	73 \pm 17

Statistical comparison of high-protein and low-protein animals among the control groups shows that their differences in weight were on the verge of being significant. The difference in gain in weight between high-fat high-protein and high-fat low-protein groups was 62 grams. The low-fat high-protein rats on an average gained 44 grams more than those on the low-fat low-protein diet. The critical ratios of these differences according to their standard deviations are all 1.8.

A similar comparison among the exposed groups shows that their differences

were very significant. The low-fat high-protein rats gained 85 grams more than those on the low-fat low-protein diet. The difference between high-fat high-protein and high-fat low-protein groups was 101 grams; their critical ratios are 3.2 and 2.9 respectively.

By all the above comparisons it is clear that exposure to benzene vapor had aggravated the effect of protein deficiency. This fact is illustrated in figure 3 where two rats are shown that were fed on the same diet—high-fat low-protein—but one had been exposed to benzene vapor. This picture was taken three days before the end of the experiment. The increased susceptibility of the protein-deficient rat to benzene poisoning is illustrated in figure 4 where two rats are shown that had been exposed to benzene vapor for eleven and a half weeks. One was fed on the high-fat low-protein diet and the other on the high-fat high-protein diet.

Varying the fat-content of the diet did not exert much influence on the growth of the control animals. The two exposed high-protein groups grew at the same rate. The growth curves of the two low-protein control groups were also almost



Fig. 3. Left - Rat on high-fat low-protein diet and exposed to benzene. Right - Rat on high-fat low protein diet without exposure.

Fig. 4. Left - Rat on high fat low protein diet and exposed to benzene. Right - Rat on high fat low-protein diet and exposed to benzene.

identical. Though the high-fat high-protein control animals gained more weight than those on the low-fat high-protein diet and though the final weight of the high-fat low-protein group with exposure was less than that on the low-fat low-protein diet, yet neither of these differences was of any significance statistically. In comparing the two corresponding groups on the same diet, one of the two high-fat diets and one of two low-fat diets had significant differences in weight-gain. However this was due to the effect of protein-deficiency as discussed above. The difference (44 grams) between the exposed and the control groups on the high-fat high-protein diet, though not significant, cannot be entirely neglected. Perhaps, the high fat content of a diet does retard the growth of animals exposed to benzene vapor, but it would be necessary to have a very large number of animals to reveal such a small difference statistically.

The growth curves of protein-deficient animals reveal the following facts: first, benzene had its effect from the very beginning of the exposure. The control low-protein rats grew at their normal rate on the diet for at least half a week before they levelled off. The growth rate of protein-deficient exposed groups was retarded immediately after the exposure and actually they did not maintain their weight in the following few weeks. Second, these animals

made some adjustment in the mechanism of detoxication after a few weeks of exposure to the vapor as evidenced by a gain in weight, after which their growth again declined.

Leucocytes. Average of the last three white counts of different groups and number of individual instances when the count fell below 10,000 are shown in table 3. All the control animals had relatively high leucocyte counts. This may have been due to the wide variation in room temperature which occurred during the course of this experiment. Though the exposed rats were subjected to the same fluctuation in the same room, yet they distinctly had fewer white cells in their circulation. The high-fat high-protein and high-fat low-protein groups with exposure had lower white counts. At the end of the experiment the the latter group had only 9,500 white cells per cu. mm. of blood. The incidence

TABLE 3
Leucocytes per cu. mm. of blood

DIETS	AVE. LAST THREE WHITE COUNTS		NO. OF INSTANCES < 10,000 W.B.C.	
	Exposed	Control	Exposed	Control
Low-fat high-protein.....	12,200	18,900	12	0
High-fat high-protein.....	10,300	24,400	13	0
Low-fat low-protein.....	12,000	18,000	17	0
High-fat low-protein.....	9,500	18,400	15	0

TABLE 4

DIETS	HEMOGLOBIN (GM. PER 100 CC. BLOOD)	
	Exposed	Control
Low-fat high-protein.....	15.7	15.4
High-fat high-protein.....	14.6	15.1
Low-fat low-protein.....	13.9	13.9
High-fat low-protein.....	13.1	12.6

of counts below 10,000 in the animals of the low-protein exposed groups was higher than for those on the high-protein diet. Counts this low were never observed in any of the control animals at any time. These facts indicate that leucopenia was due to benzene-poisoning and that protein-deficiency aggravated the effect.

Hemoglobin. Averages of the last three hemoglobin determinations for the different groups are shown in table 4. Anemia occurred in all low protein groups whether exposed or not. There was no difference between corresponding exposed and control groups. Fat content of the diets had no significant effect on hemoglobin, though the low-fat low-protein groups had slightly higher values than high-fat low-protein groups. It can be concluded that the anemia was caused by protein-deficiency and uninfluenced by exposure to benzene.

Liver lipids. The concentration of total lipids in the pooled livers from various

groups are presented in figure 5. The liver lipids of the high protein groups, whether exposed to benzene or not, were within normal range and were all nearly the same. The low-protein groups all had some degree of fat infiltration. The livers from the high-fat low-protein animals contained more lipids than those on the low-fat low-protein diet. All the controls of the two low-protein diets had higher percentage of liver lipids than the corresponding exposed groups. This may be explained by the difference in growth as in the case of limitation of thiamine intake described by McHenry (6) or in minerals as reported by Handler (7). The difference in weight gain was 55 grams on the low-fat low-protein diet and 83 grams on the high-fat low-protein diet. The size of the liver calculated as gram per 100 grams body weight was about the same except that the low-protein control groups were slightly (0.7 gram) heavier than those of the corresponding exposed groups.

Food intake. The average number of calories consumed daily by individual rats of different groups at the beginning of the experiment were essentially the same. On the high-protein diets, the food intake of the exposed and the

TABLE 5
Average daily individual food intake during the last three weeks

DIETS	EXPOSED		CONTROL	
	Total Cal.	Cal./100 gm. body wt.	Total Cal.	Cal./100 gm. body wt.
Low-fat high-protein.....	44.0	16.4	45.7	16.5
High-fat high-protein.....	42.0	15.7	45.6	14.9
Low-fat low-protein.....	32.6	17.9	40.5	17.1
High-fat low-protein.....	25.9	16.9	44.9	18.8

control groups, except certain fluctuations, were approximately equal throughout the entire period. The high-fat low-protein exposed group began to consume less food than the corresponding control group, after three weeks of exposure. The difference in the low-fat low-protein groups began after another two weeks of exposure. With the exception of these two groups, the caloric intake of all rats showed no significant difference at the end of the experiment. The average food intake during the last three weeks is shown in table 5. The food intake per unit of body weight of rats of different sizes, was inversely related to their size. The small rats consumed, as one might expect, more food per unit of body weight than the bigger ones. Their restricted growth was probably due to the necessity of using sulphur for detoxication which was needed for growth. It has been shown that the growth of rats given brombenzene is limited by the intake of sulphur-containing amino acids (8).

One peculiar practice noticed in this experiment was that all the high-fat low-protein rats, whether exposed to the solvent or not, chewed off the edges of their food cups. These cups were made of Monel metal. The results of this practice were noticeable after the sixth week and some cups had very deep notches along the edge by the end of the experiment.

GENERAL COMMENT. Rats seem to be less susceptible to benzene poisoning than dogs under a similar protein restriction. Though the high-fat low-protein exposed animals appeared to be sick, yet the protein-deficient rats all survived the effect of twelve weeks of exposure. One of four dogs on a similar low-fat low-protein diet and three of five on a similar high-fat low-protein diet died in a shorter period of time. Two of four dogs of the former group and four of five of the latter group had leucopenia before the tenth week of exposure (1). Rats on similar diets had less leucopenia.

Though rats are more resistant to benzene poisoning than dogs, yet an inadequate protein intake affects the susceptibility of both species. A high protein intake supplies more abundantly elements such as sulphur that are necessary for the mechanism of detoxication. In this report as well as in the experiment with dogs (1) the fat content of diets seemed to have some influence on susceptibility to benzene poisoning. There was some ill-effect on a high-fat diet, especially in the case of a low-protein content, both in dogs and in rats. This experiment emphasizes the point also shown to be true for dogs that exposure to benzene that may be well tolerated when animals are on an adequate diet, may produce harmful effects if the protein intake is restricted.

Himsworth and Glynn (9) studied the relation of diet to the toxicity of T.N.T. which is a closely related compound of benzene. They attributed the relatively severe symptoms and marked pathological lesions in T.N.T. rats taking a high fat diet only to the "high-fat" content, but failed to emphasize the difference in protein intake among different groups. Their "protein diet" contained about 60 per cent of protein, while their "fat and carbohydrate diet" contained only about 13 per cent protein. The protein per 100 Calories was much higher in their "carbohydrate diet" than in their fat diet.

SUMMARY

1. The protein content of the diet is of primary importance in determining the susceptibility of rats to benzene poisoning.
2. Exposure to 600 P.P.M. by volume of benzene for 42 hours per week further retarded the growth of rats fed a protein-deficient diet.
3. Rats exposed to benzene and maintained on a high-fat diet manifested a greater incidence of leucopenia than do control groups, irrespective of the protein content of the diet.
4. Rats on a protein deficient diet were less susceptible to benzene poisoning, as judged by mortality and degree of leucopenia, than are adult dogs maintained on comparable diets.

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THE INCREASED SUSCEPTIBILITY OF PROTEIN-DEFICIENT DOGS TO BENZENE POISONING

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In a previous communication (1) it was demonstrated that dogs are quite resistant to chronic benzene poisoning when maintained on an adequate stock diet. It may be assumed that the basis for this resistance is common to other species, including man, and that information contributing to an understanding of the factors which determine the dog's resistance may further the recognition of the factors which influence the susceptibility of human beings to benzene. For numerous reasons it seems plausible to suppose that the protein content of the diet and the functional state of the liver may both be of primary importance in determining the body's capacity to detoxify benzene. However, a search of the literature failed to reveal any study which demonstrated the effect of protein deficiency upon the susceptibility of animals to benzene poisoning. Studies were then undertaken to determine the effect of an inadequate protein intake upon the tolerance of dogs for inhaled benzene. Observations were also made upon the effect of varying the fat content of the diet on susceptibility to benzene fumes. Two species were used, dogs and rats; the present communication deals with observations made on dogs.

METHODS AND DIETS. Eight adult male dogs were divided into four groups. Each group was fed with one of four different diets supplying 2.0 or 0.8 gram of casein, 2.6 grams or 1.4 grams of fat and 40 cal./lb. of body weight daily except Sunday. The composition of these diets is shown in table 1.

The animals were exposed to commercial benzene (90 per cent C_6H_6) at 600 p.p.m. by volume for 42 hours per week in the exposure chambers described in the previous communication (1), after they had been on the diets for three weeks. Two such chambers were used, each holding four dogs, a dog from each group was put in one of the two tanks so as to compensate for any slight variations in the concentration of the solvent in the two chambers. Animals that died were replaced with new dogs which had been on the same diet for three weeks. Thus, throughout the experiment there were two dogs in each dietary group and one from each group in each chamber. Two additional dogs were fed a diet like the high-fat one listed in table 1, except that casein was entirely omitted, and were exposed to benzene vapor in the same manner. Two other dogs were fed the high-fat low-protein diet without exposure. All animals were weighed every week.

Hemoglobin concentration, erythrocyte and leucocyte counts were determined at weekly intervals during the entire period. Blood samples were drawn immediately after exposure before feeding on Monday evenings. In the latter

part of the experiment, blood was also drawn on Tuesday mornings, before exposure, for thrombocyte counts.

The condition of the livers was evaluated at two-week intervals by the Rose Bengal dye clearance test using the method of Stowe, Delprat and Weeks (3) with modifications described by Hough and Freeman (4). Serum phosphatase was estimated at the same time by Bodansky's method (5) during the latter part of the experiment. The determination of phosphorus was made by Gomori's modification of Fiske-Subbarow's method (6).

Urine specimens were collected after the exposure on Thursday evening until Friday morning before exposure, using chloroform as the preservative. The concentration of total and free sulphates was determined by the method of Folin (7).

TABLE 1
Composition of various diets

	LOW-FAT HIGH-PROTEIN (LH)*	HIGH-FAT HIGH-PROTEIN (HH)*	LOW-FAT LOW-PROTEIN (LL)*	HIGH-FAT LOW-PROTEIN (HL)*
Lard (%).....	15	33	15	33
Casein (%).....	21	25	8.5	10
Sucrose (%).....	53	30	65.5	45
Wesson's salt mixture (2) (%).....	2	2	.	2
Dried brewer's yeast (%).....	4.5	5	4.5	5
Cellophane (%).....	4.5	5	4.5	5
Percomorph oil (drops per kgm.).....	9	10	9	10
Amount fed daily (gm. per lb. body wt.).....	9.5	8.0	9.5	8.0
Fat supplied (gm. per lb. body wt.)...	1.4	2.6	1.4	2.6
Protein supplied (gm. per lb. body wt.).....	2.0	2.0	0.8	0.8

* These symbols will be used in all tables.

Animals were sacrificed when they became moribund and were immediately autopsied. Dogs still alive at the end of a year were sacrificed. Samples of tissues were preserved for microscopic examination and livers were analyzed for their lipid content by the method of Best, Channon and Ridout (8).

RESULTS AND DISCUSSION. *Survival period.* The survival period of all dogs is tabulated in table 2 together with the change in body weights during the entire period of exposure. The animals on the low-protein diet died much sooner than those receiving two grams of casein per pound body weight daily. The former groups survived five to thirty-six weeks; while in the latter groups, survival varied from forty-three weeks to those which were still in good condition at the end of a year of exposure. The difference cannot be accounted for merely by the effect of inadequacy of protein intake. This is demonstrated by the fact that the control dogs on the high-fat low-protein diet without exposure survived forty-two to fifty-two weeks or more.

Because of the limitation in the number of animals used and wide variation in time of survival among the dogs in the individual groups, no significant difference could be found between high and low fat diets. However, there were indications that a diet rich in fat increased the susceptibility of animals to the toxicity of benzene even with two grams of protein per pound of body weight daily. Two dogs on the high-fat, high-protein diet were sick after forty-three

TABLE 2
Survival period, body weight, liver lipids and condition at the end of experiment

DIETS*	DOGS	SUR- VIVAL PERIOD	CHANGE OF BODY WT.	LIVER LIPIDS	CONDITION AT THE END
		<i>weeks</i>	<i>lb.</i>	<i>%</i>	
LH	(22V)	52+	+3½	5.9	Good, sacrificed
	(23VA)	51+	+4½	5.2	Good, sacrificed
HH	(18V)	49	+5	5.3	Sudden onset of profound weakness or paraplegia with death in 2 days
	(19V)	43	+1½	18.1	Sick, very weak, died
LL	(24V)	6	-4	22.6	Moribund, sacrificed
	(24VB)	24	-4½	10.8	Moribund, sacrificed
	(25V)	33	-2	12.4	Died during exposure
	(25VA)	36	+1	25.9	Moribund, sacrificed
HL	(20V)	27	-3½	6.0	Moribund, sacrificed
	(21V)	15	-6½	7.1	Moribund, sacrificed
	(21VA)	5	-7½	17.6	Moribund, sacrificed
	(21VB)	6	-10½	23.5	Moribund, sacrificed
	(21VC)	9	-3½	24.9	Moribund, sacrificed
HN†	(32V)	8‡	-6½	28.3	Moribund, sacrificed
	(33V)	8‡	-3½	20.1	Moribund, sacrificed
HL No exposure	(26V)	52+	+3	12.2	Normal activity, coat shabby, partial anorexia
	(27V)	42	+1½	21.2	Sick, profound weakness, collapse

* For explanation of these symbols, see table 1.

† HN = a high fat diet in which casein was entirely replaced by sucrose.

‡ No preliminary period on the diet before the exposure.

and forty-nine weeks of exposure; while two dogs on the low-fat high-protein diet were still in good condition at the end of a year. The difference between the two low-protein groups was similar. The average survival period of the low-fat low-protein group was twenty-one weeks with three living about half a year. The average for those on the high-fat low-protein diet was only twelve weeks with only one living about half a year.

Hepatic dye clearance and serum phosphatase. In table 3 are shown the results obtained by use of the dye clearance test and serum phosphatase deter-

mination. The values given for each dog are the initial and final values together with the time at which the result of the test could be considered definitely abnormal. In all animals there was some decrease in the rate at which the dye was removed from the blood stream, but the decrease was much more marked and occurred earlier in the animals exposed to benzene and re-

TABLE 3
Results of hepatic dye clearance tests and serum phosphatase determinations

DIETS*	DOG	ROSE BENGAL CLEARANCE			SERUM PHOSPHATASE		
		Initial	Last week	First time < 80 %	Initial Bodansky	Last week	First time > 5 units
LH	(22V) (23VA)	%	%	week	units	units	week
		116 98	107 100	Never 46th Once only	3.6	2.4 6.2	42nd
HH	(18V)	104	103	Never		5.7	47th
	(19V)	99	82	Never		4.3	Once only Never
LL	(24V)	106	43	4th		16.9	
	(24VB)	94	34	24th	2.8	4.3	
	(25V)	106	65	26th		4.0	10th
	(25VA)	96	80	34th Once only	1.5	4.9	Once only Never
HL	(20V)	95	66	22nd		12.4	
	(21V)	104	38	10th		9.3	
	(21VA)	105	38	3rd	1.1	4.0	6th
	(21VB)	106	61	2nd	1.7	5.2	Never
	(21VC)	100	36	9th	1.5	16.9	6th 9th
HN	(32V)	127	27	8th	1.8	11.1	8th
	(33V)	124	35	7th	1.9	12.0	7th
HL No exposure	(26V)	126	72	40th	2.2	23.3	8th
	(27V)	92	49	38th	1.9	17.9	10th

* For explanation of these symbols, see table 1 and table 2.

ceiving low-protein diets than in either the group receiving high-protein diets plus benzene exposure or the controls receiving the same low-protein diet without exposure to the solvent. The serum phosphatase also became abnormal earlier in the animals exposed to benzene. The maximum change in enzyme concentration was no greater in the exposed animals probably because of their shorter survival period.

It is interesting to note that the abnormality in serum phosphatase of the two control animals came much earlier than the decline of the hepatic dye

clearance. It seems that moderate inadequacy in dietary protein gives a different picture of liver impairment from that of extreme protein deficiency (4, 9).

Leucocytes and thrombocytes. The initial values, the averages of the last three counts, the lowest figures during the entire period of exposure and the time when definite abnormality occurred in white cell and platelet counts are combined

TABLE 4
Leucocyte and thrombocyte counts

DIETS*	DOGS	LEUCOCYTES					THROMBOCYTES				
		In- initial	Average last 3 counts	Lowest		First time < 8,000	In- initial	Average last 3 counts	Lowest		First time < 100,000
				Fig- ure	When				Figure	When	
					week	week				week	week
LH	(22V)	11,900	11,600	5,500	33rd	26th (up again many times)		148,000	100,000	48th	Never
	(23VA)	16,200	10,200	8,600	51st	Never	175,000	34,000	14,000	51st	39th
HH	(18V)	14,150	15,500	7,400	31st	31st (only once)		137,000	85,000	39th	39th (only once)
	(19V)	12,700	5,100	3,500	31st	7th (down most of the time)		90,000	70,000	39th	39th (up again a few times)
LL	(24V)	13,700	4,500	250	7th	4th	220,000	182,000	50,000	20th	20th (up again)
	(24VB)	18,800	4,900	5,000	21st	8th					
	(25V)	11,500	5,900	3,800	28th	13th (up again 3 times)					
	(25VA)	16,400	5,400	4,700	36th	22nd	340,000	3,000	0	26th 34th 36th 37th	21st (up again a few times)
HL	(20V)	10,500	1,200	850	27th	6th (down most of the time)					
	(21V)	12,400	7,200	5,200	13th	10th	290,000 190,000 190,000	37,000 67,000 227,000	0 0 140,000	4th 6th 9th	3rd 6th Never
	(21VA)	12,700	12,800	10,200	3rd	Never					
	(21VB)	13,400	4,500	3,500	6th	6th					
	(21VC)	12,400	6,500	4,600	9th	7th					
HN	(32V)	14,900	9,500	7,450	7th	6th	190,000	49,000	43,000	8th	8th
	(33V)	16,900	10,300	9,400	6th	6th	170,000	153,000	100,000	8th	Never
HL No expos.	(26V)	14,800	38,000	11,600	8th	Never	260,000	226,000 373,000	75,000 102,000	33rd 28th	33rd (only once) Never
	(27V)	12,000	26,500	10,900	7th	Never					

* For explanation of these symbols, see table 1 and table 2.

in table 4. The lower limits of normality were arbitrarily set at 8,000 for the leucocytes and 100,000 for the thrombocytes. The control dogs on the high-fat low-protein diet without exposure showed a very wide variation in their counts but maintained high values throughout the experiment with one exception when one animal once had a platelet count of 75,000. The effect of benzene poisoning in reducing the leucocyte and thrombocyte count was manifested in nearly all exposed animals.

That the leucopenia was much more marked in protein-deficient animals is shown by the averages of the last three counts of individual animals and by their lowest figure. In two high-fat low-protein exposed dogs counts were obtained showing only 250 and 850 white cells per cu. mm. in their blood. The thrombocytopenia produced by benzene inhalation was more common on the protein-deficient diets and most frequently occurred in animals with a leucopenia although these two effects were not necessarily maximal at the same time. The blood of three dogs on the low protein diets was on several occasions reported as "no platelet found". Usually, such findings were repeated as soon as possible, and they were considered established only when they were followed by a similar report or by a very low value. There was no difference between the high and the low fat groups with the same protein intake.

Erythrocytes and hemoglobin. Red cell counts and hemoglobin determinations are to be found in table 5. Five million red cells per cu. mm. of blood and 13 grams per cent hemoglobin are considered—again arbitrarily—as the lower limits of normal. Of the fifteen animals used all those which went below normal were in the benzene-exposed, low-protein groups; in some of these the anemia was rather marked. Not all, however, showed a significant decrease in red cell count or hemoglobin content.

One cause of anemia in these animals is an inadequate protein intake, but the process must have been hastened and augmented by benzene poisoning. Not only were the final red count and hemoglobin content lower than the controls but the reduction was also much more rapid. Several of the low-protein exposed dogs never had values below the lower limits of normal, hence anemia was not as constant a manifestation of benzene poisoning as leucopenia. Since no blood volume studies were made, some degree of anemia may have been obscured by hemoconcentration.

Conjugated sulphates and other findings in urine. Adequate means for collecting urine in the exposure chambers were not available. Since twenty-four hour specimens were not obtainable, only the percentages of conjugated sulphates are considered. The results of the urinary analysis of the preliminary period, of the first five weeks after exposure, and the average of the final period are summarized in table 6. If animals survived more than twenty weeks the results of the last ten weeks were averaged. Otherwise, figures for the last half of the survival period were used to obtain the average value.

It is clear that conjugation with sulphates is one of the important mechanisms in the process of detoxication. The percentages of conjugated sulphates in the urine of control dogs remained as low as they were before the experiment. The severity of benzene intoxication seems to have been related to the percentage of conjugation of sulphates. The exposed animals which remained in good condition to the end of the experiment excreted much more sulphates in a free form than those that succumbed to the effects of benzene. With the exception of 23VA all high-protein animals had less than 82 per cent of conjugation in excreted sulphates; while all low-protein dogs had 85 per cent or more.

Although data on the daily total amount of sulphate excreted was not ob-

tainable, it appears from the concentration in the overnight samples that the low-protein animals excreted sulphates in lesser amounts and that the amount of free sulphate decreased as exposure proceeded, becoming very small at the

TABLE 5
Erythrocyte counts and hemoglobin determinations

DIETS*	DOGS	ERYTHROCYTES (MILLION PER CU. MM.)					HEMOGLOBIN (GRAMS PER 100 CC.) gm.				
		Initial	Ave. Last 3 counts	Lowest		When < 5.00	Initial	Ave. Last 3 detmn.	Lowest		When < 13
				Figure	When				Value	When	
					week	week				week	
LH	(22V)	6.68	6.14	5.54	50th	Never	14.6	14.3	12.3	2nd	2nd (only twice, up again)
	(23VA)	5.42	6.57	5.52	10th	Never	13.1	14.7	13.0	2nd	Never
HH	(18V)	6.43	6.66	6.08	43rd	Never	14.6	15.5	13.9	42nd	Never
	(19V)	7.53	7.63	6.31	7th	Never	16.1	17.4	15.6	5th	Never
LL	(24V)	6.85	4.49	3.35	7th	6th	15.1	10.7	8.8	7th	2nd
	(24VB)	5.74	4.75	3.88	24th	24th	13.3	9.2	7.8	24th	20th
	(25V)	5.29	6.45	5.04	8th	Never	13.5	14.8	12.2	5th	5th (up again, final drop)
	(25VA)	6.54	3.57	1.67	37th	34th	15.2	10.5	4.6	37th	36th
HL	(20V)	7.55	4.42	3.96	27th	27th	14.4	7.7	6.3	29th	9th
	(21V)	7.26	4.27	3.47	15th	15th	14.7	10.7	9.8	15th	11th
	(21VA)	7.45	6.85	6.25	5th	Never	16.5	16.5	15.3	5th	Never
	(21VB)	7.73	5.91	5.05	6th	Never	15.0	12.4	11.0	6th	6th
	(21VC)	7.05	7.20	6.52	9th	Never	15.4	17.2	14.5	5th	Never
HN	(32V)	6.64	7.12	6.30	5th	Never	15.5	17.5	15.8	3rd	Never
	(33V)	7.21	7.22	6.16	1st	Never	16.0	17.8	17.8	2nd	Never
HL No exposure	(26V)	7.79	6.68	6.16	41st	Never	17.0	15.4	14.1	18th	Never
	(27V)	6.21	5.58	5.22	39th	Never	15.1	13.8	13.0	39th	Never

* For explanation of these symbols, see table 1 and table 2.

end. These findings suggest that the protein-deficient animals lacked available sulphur for further detoxication.

The average results of the first five weeks and the figures for the fifth week of exposure of high and low protein groups were distinctly different. The total average of five weeks of the former groups was 47 per cent with the fifth week at 62 per cent, while the average of those of the latter groups was 78 per cent with the fifth week at 77 per cent. Animals on a high-protein diet can supply more protein for metabolism and thus more sulphate is available for detoxifica-

tion. Perhaps the above differences in percentage conjugation merely reflect a difference in availability of sulphur.

It is of interest to note that an alteration in the percentage of conjugated sulphates in the urine appeared much earlier than any other findings in chronic benzene poisoning. As Yant et al. (10) have pointed out, this change can serve as an indicator of the absorption of benzene vapor.

TABLE 6
Percentage of conjugated sulphates in urine

DIETS*	DOGS	PRELIMINARY PERIOD (%)				FIRST 5 WEEKS OF EXPOSURE (%)						AVERAGE OF LAST 10 WEEKS OR LAST HALF OF TIME OF EXPOSURE† (%)
		1	2	3	Ave.	1	2	3	4	5	Ave.	
LH	(22V)	1	5	5	4	18	17	25	21	39	24	50
	(23VA)	4	2	3	3	81	62	92	85	89	82	93
HH	(18V)	1	4	2	2	29	33	34	50	72	44	65
	(19V)	8	7	4	6	45	36	32	40	49	40	82
LL	(24V)	6	6	9	7	50	67	82	94	89	76	91 (3)
	(24VB)	5	7		6	73	82	92	94	95	87	89
	(25V)	12	9	4	8	63	57	62	54	85	64	85
	(25VA)	9	7	7	8	42	53	91	54	5	59	89
HL	(20V)	5	19		12	56	54	47	55	90	60	88
	(21V)	6	11	11	9	83	75	74	80	87	80	87 (8)
	(21VA)	8	11	5	8	79	79	92	95	94	88	94 (3)
	(21VB)		6	7	4	94	94	95	94	90	93	92 (3)
	(21VC)	9	9	7	8	18	48		53	93	53	88 (6)
HN	(32V)			10‡	10‡	80	74	93		95	85	92 (4)
	(33V)			14‡	14‡	72	73	74	84	96	80	95 (4)
HL No exposure		(First 3 weeks)				(Succeeding 5 weeks)						(Last 10 weeks)
	(26V)	3	6	8	6	5	13	8	6	5	7	8
	(27V)	7	5	8	7	7		7	5	4	6	8

* For explanation of these symbols, see table 1 and table 2.

† The figure in parenthesis, following percentage, indicates number of weeks included in the average.

‡ Results obtained on stock diet.

The urine of most of the exposed dogs near the end of their survival period was very dark in color; some specimens contained bile pigments and protein.

Liver lipids. The lipid content of the fresh livers is listed in table 2. All low-protein animals showed more or less fatty infiltration whether they had been exposed to benzene vapor or not. Their lipid content varied from 10.8 to 25.9 per cent. One dog on high-fat high-protein diet had 18.1 per cent lipids in its liver. Other high-protein animals had a normal concentration of lipids in their

livers. Variation in the fat content of the diet could not be correlated with fat content of the liver.

Microscopical examination of tissues. With the exception of a few accidental omissions, examination of sections from the heart, aorta, liver, kidneys and bone marrow were made from each animal in this series. The adrenals, thyroid, spleen, pancreas and lung were examined less systematically. Paraffin sections were stained routinely with hematoxylin and eosin. Frozen sections of the livers were stained with sudan IV or oil red O. In studying sections of the bone marrow, no attempt was made to estimate accurately the relative number of marrow cells of various types. Since there was no record of the ages of the dogs, small differences in degree of cellularity or in proportions of different types of cells would have no significance for the experiment. Therefore only major deviations from normal were looked for. Rough estimates were made of the degree of cellularity of the marrow from vertebral bodies, sternum and ribs. Only the findings in the liver and bone marrow will be included in this report as other changes were incidental and apparently unrelated to the experimental procedures.

Liver. In the livers of three dogs (18V, 22V and 23VA), the appearance in routine paraffin sections and the quantity of fat estimated from stained frozen sections were entirely normal. All of the others showed some degree of vacuolation and distortion of liver cells and increased quantities of stainable lipids, graded as 1 plus to 4 plus (1 plus being considered as normal). The estimates of stainable fat were roughly proportional to the content of liver fat by chemical analysis and do not need to be enumerated in detail since the results of chemical determination are given in table 2. The distribution of the fat in sections was essentially the same in all cases and appeared most abundant in the intermediate and central zones of the liver lobules. The most extreme distortion of the cells by fat globules was invariably found in the intermediate zones. In a few samples, the only parenchyma cells with normal appearance were found in a narrow zone around the periportal connective tissue. Increased quantity of connective tissue was present in the liver of only one dog (25VA). In this case there was a finely nodular cirrhosis with slender trabeculae subdividing the liver into pseudolobules of various sizes. The presence of numerous small and large areas of necrosis with leucocytic infiltration and fibrinoid masses indicated that the process was active.

Bone marrow. All sections of marrow except one (24V) showed normal proportions of cells of the two principal types or a disproportionate increase in number of granulocytes. The estimated degree of cellularity was roughly correlated with the length of survival of the animal, those dying or sacrificed within five to nine weeks showing normal or slightly increased degrees of cellularity and those surviving for longer periods showing abnormally cellular marrows with fat reduced to as low as 5 per cent of the areas of marrow spaces represented by the sections. The general trend was toward hyperplasia of granulocytes with evidence of increased rate of maturation (disproportionate increase of young polymorphonuclear neutrophils). In most samples the relative number of cells was greater in ribs and sternum than in the vertebral bodies.

In two specimens the degree of cellularity was estimated to be less than normal. Dog 24V (six weeks survival period) showed hypoplasia of marrow cells with predominance of normoblasts and suppression of mature or maturing granulocytes. In the marrow of the other dog (20V) in which diminished cellularity was observed, normoblasts were abnormally few and immature granulocytes (band forms, juveniles and myelocytes) predominated. Ninety per cent to ninety-five per cent of the area of the marrow spaces consisted of reticular stroma, dilated sinusoids, protein-filled spaces and fat vacuoles in the reticulum. The fat content of the marrow was much reduced. This dog had survived 27 weeks of experimental procedures and had a mild degree of bronchopneumonia at death.

General comments. The results of all the observations clearly demonstrate that the level of protein intake influences the susceptibility of animals exposed to benzene. Benzene exposure that was not harmful to an animal with an adequate protein intake uniformly caused toxic manifestations to others on a protein-deficient diet. It is reasonable to suppose that this finding applies to human beings since the means of detoxifying benzene are similar in the two species.

The effect of protein deficiency on the toxicity of benzene may not only be due to the absence of an adequate source of sulphur to conjugate with the oxidation products of benzene, but also to impairment in other means of detoxication which depend upon the function of the liver. The adverse effect of fat may be due to its solvent action on benzene or to further impairment in liver function which it produces. The displacement of carbohydrates from the diet by fat may also be a factor in the latter effect. With a fixed intake of protein, an increase of fat in the diet must be accompanied by a decrease in carbohydrate consumption. This may interfere with the glycogen reserve which is of consequence since glycogen may be the precursor of glucuronic acid which is also used in the organism to conjugate with benzene derivatives (11, 12).

Among the findings on protein-deficient dogs exposed to benzene are nearly all of the manifestations reported as occurring in human beings with benzene poisoning, namely, leucopenia, agranulocytosis, anemia, thrombocytopenia, gastrointestinal bleeding, jaundice, hepatic insufficiency and hyperplasia, and aplasia of the bone marrow. Differential counts were not consistently made as a part of this study. However, the myelogenous tissue was found to be aplastic in two animals with marked leucopenia and differential counts on these animals revealed an almost complete absence of granulocytes from the peripheral blood.

SUMMARY

1. An inadequate protein intake (0.8 gram per lb. per day) markedly increases the susceptibility of dogs to chronic benzene poisoning.
2. A high-fat low-protein diet caused the highest mortality and shortest survival period when dogs were exposed to 600 p.p.m. by volume of benzene for 42 hours per week.
3. Protein deficiency increases the percentage conjugation of inorganic sulphates in the urine of dogs exposed to 600 p.p.m. by volume of benzene.

4. Most of the clinical characteristics and laboratory findings of benzene poisoning as reported for human subjects can be reproduced in dogs maintained on a protein-deficient diet and exposed to benzene fumes.

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THE EFFECT OF ACETYLCHOLINE ON THE ATROPINIZED, DENERVATED HEART¹

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The administration of acetylcholine to the normal dog causes an abrupt fall of blood pressure due to a brief depression of the heart, often accompanied by A-V heart block, and probably also to vasodilatation. As shown by Youmans, Aumann, Haney and Wynia (3) there results a strong activation of those compensatory mechanisms which are designed to return the blood pressure toward normal. They include marked tachycardia induced by reflex excitation of the sympathetic cardio-accelerator fibers, the adrenal medulla and probably vagal cardio-accelerator fibers (1) as well as depression of cardio-inhibitory fibers of the vagus and the production of sympathin. In addition to these factors it is necessary to consider the possibility that acetylcholine exerts a direct stimulatory action on the heart tissues or on ganglion cells within the heart, an action independent of and in opposition to its cardio-depressor influence.

METHOD. Dogs were subjected to removal of the stellate and upper 5 or 6 thoracic sympathetic ganglia and section of both vagus nerves. In three cases the adrenals were demedullated or denervated. The animals were then trained to lie quietly while submitting to venipuncture and electrocardiographic recordings without use of anesthesia. At the beginning of each experiment, atropine sulfate was administered intravenously in a dose of 2.6 mgm. ($\frac{1}{2}$ grain). After an interval of 10 to 20 minutes, a 22-gauge needle attached through a 3-way stopcock to a syringe filled with saline was introduced into the vein of the front leg. The continuous electrocardiographic tracing then was started. After about 30 seconds of recording, acetylcholine in doses of 1 to 4 mgm. was injected rapidly from another syringe attached to the stopcock, and the recording continued for the desired time interval.

The heart rate was determined before and during 5-second periods after the injection of the acetylcholine. The point of onset of acceleration was determined by means of a caliper.

RESULTS. In all but 4 of 25 experiments performed on 8 dogs the heart rate increased more than 11 beats per minute in response to injection of the acetylcholine, as shown in table 1. The time interval between onset of injection of acetylcholine and the onset of acceleration varied from 4 to 7 seconds.

There exists a degree of correlation between the dose of acetylcholine and the magnitude of increase in heart rate. Thus in the 4 experiments on 4 of the dogs in which the dose was 1 mgm. the increase ranged from 4 to 78 beats per minute, 2 showing a rise of only 4 beats per minute. In the 10 experiments on 8 dogs in

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which the dose was 2 mgm. the increase ranged from 2 to 130 beats per minute, the average was 46 and in 5 of the 10 experiments there was a rise of 60 or more.

TABLE 1

Effect of acetylcholine on the denervated heart following intravenous injection of 2.6 mgm. of atropine sulfate

The adrenals had been demedullated in dogs 1, 2 and 3, and denervated in dog 4.

DOG NO.	WEIGHT	DOSE OF ACETYLCHOLINE	HEART RATE PER MINUTE										TIME BETWEEN INJECTION OF ACETYLCHOLINE AND ONSET OF ACCELERATION
			Before injection of acetylcholine	During 5-second intervals following injection of acetylcholine									
				1	2	3	4	5	6	7	8		
	kgm.	mgm.										sec.	
1	12.3	1	180	180	184	184	182	184	182	180		7 6	
		2	180	186	192	188	188	186	186	184			
		4	180	210	246	240	228	210	204				
2	17.0	2	156	156	210	234	210	192	174	168		6	
		4	154	204	228	210	186	180	168	160	6		
3	9.1	1	182	182	180	186	186	.				5 5	
		2	178	180	198	204	198	196	192	192			
		4	180	184	222	240	228	218	210	204			
4	14.0	2	122	124	124	124	124	124	124	122		6 6	
		4	120	122	132	132	128	128	126	124	124		
		2	132	134	134	134	134	132	132	132			
		4	132	132	156	150	138	144	144	140	140		
5	12.0	2	112	114	128	132	*	186	168	170	160	6	
		4	118	120	166	166	*	*	260	270	264	6	
		2	112	112	144	216	234	242	240	234	218	6	
		4	112	112	150	240	350	346	348	336	336	6	
6	15.0	2	108	108	140	174	174	180	174	170	168	6	
		4	102	102	162	180	210	222	228	228	228	5	
		4	106	114	150	160	200	216	216	222	222	4	
7	9.5	1	138	138	160	204	204	204	196	184	180	5	
		2	144	144	156	176	172	174	176	174	172	6	
		4	140	138	174	210	258	252	254	252	252	5	
8	10.9	1	130	136	188	208	204	186	174	162	156	5	
		2	136	136	156	212	218	208	204	198	192	6	
		4	150	156	228	240	258	266	264	264	246	4	

* Dog struggled.

In the 11 experiments on 8 of the dogs in which the dose was 4 mgm. the increase ranged from 12 to 238 beats per minute, the average was 99 and in 9 of the 11 experiments there was a rise of 60 or more.

DISCUSSION. Atropine sulfate in the dosage indicated prevents acetylcholine from acting to produce direct depression of the heart and vasodilatation. As a consequence the fall of blood pressure normally produced by acetylcholine does not occur. The cardio-acceleration which results from the administration of acetylcholine to the atropinized dog therefore cannot be explained on the basis of a fall of blood pressure and the resulting reflex excitation of the mechanisms which operate to compensate for such a fall.

The fact that the hearts had been completely denervated according to usually accepted methods is a basis for the exclusion of the extrinsic nerves of the heart as a factor in the production of the acceleration. There remain three possible factors the first of which is humoral and concerns adrenine and sympathin. It is possible that acetylcholine might circulate to the ganglion cells or centers of the sympathetic nervous system and to the cells of the adrenal medulla and excite them. Atropine of course does not abolish the nicotinic action of the compound. The result conceivably could be a discharge of adrenine and of sympathin into the venous circulation which in turn would carry them to the heart. This mechanism would require the time intervals involved in the transport of acetylcholine from the point of injection in the fore limb to the right side of the heart, through the pulmonary circuit to the left side of the heart, and through the systemic arteries to the sympathetic nerve cells concerned or to the adrenal. In addition it would require the time interval for the transport of these hormones from their site of origin through the veins to the heart. It is very unlikely that the total time involved would be as brief as 7 seconds, the greatest interval between injection of acetylcholine and the onset of tachycardia observed in our experiments.

As indicated in table 1, the time interval between injection of acetylcholine and onset of acceleration varied between 4 and 7 seconds. The brevity of these time intervals is strong evidence against the possibility that adrenine and sympathin account for the cardio-acceleration at its onset. The continuation of the tachycardia however may have its source in part in such humoral mechanisms. Of interest in this connection is the fact that demedullation of the adrenals, as indicated in table 1, does not abolish the response.

A second possible cause of the cardio-acceleration concerns a direct pharmacological action of acetylcholine on the atropinized heart. Such an action can be presented only as a possibility for which no direct evidence is available.

A third explanation concerns the possibility that acetylcholine may stimulate intracardiac ganglion cells whose function is cardio-acceleration. Such ganglion cells have not been described in relation to the sympathetic division of the autonomic nervous system. Although they commonly are believed to be parasympathetic and cardio-inhibitory, it is possible that some of them may have a cardio-accelerator function, i.e., they may be adrenergic rather than cholinergic. This possibility is supported by several investigators who have presented evidence in favor of cardio-accelerator fibers in the vagus nerves (1, 2). It seems probable that the ganglion cells of such an accelerator mechanism would be located in the heart.

It seems unlikely that direct stimulation of intracardiac axones of the sympathetic division of the autonomic nervous system accounts for the acceleration. These fibers almost certainly have undergone degeneration during the period of more than two weeks which elapsed between the sympathectomy operations and the performance of experiments.

SUMMARY

Dogs having denervated hearts were given 2.6 mgm. of atropine sulfate intravenously. The response of the heart to doses of 1, 2 and 4 mgm. of acetylcholine was determined by means of the electrocardiograph. In 21 of 25 experiments on 8 dogs the heart rate increased more than 11 beats per minute in response to the acetylcholine. The increase ranged as high as 238 beats per minute. The time interval between injection of the acetylcholine and onset of tachycardia varied from 4 to 7 seconds. The brevity of this time interval is in favor of a direct stimulatory action of acetylcholine either on the atropinized heart tissue or on intracardiac ganglion cells having a cardio-accelerator function.

Addendum. Following the submission of this report, an article on the stimulating effect of acetylcholine on the mammalian heart by Hoffmann, Hoffmann, Middleton and Talesnik has appeared in *THIS JOURNAL* **144**: 189, 1945. In a very careful study using the heart-lung and the Langendorff preparations clear-cut evidence was obtained that acetylcholine stimulates the atropinized heart. Evidence is given also for the liberation of an epinephrine-like substance in the perfusate collected following administration of acetylcholine. The findings of these workers and the results of our studies are mutually confirmatory.

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CONSIDERATION OF THE MECHANISM OF NEUTRALIZATION OF ENDOGENOUS GONADOTROPHIC HORMONE OF THE RAT BY ANTIGONADOTROPHIC SERUM¹

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Ample evidence has been presented to show that inhibition of endogenous gonadotrophic secretion may follow the administration of antigonadotrophic serum (Parks and Rowlands, 1936; Rowlands, 1937; Collip, 1937; Kupperman, Meyer and Hertz, 1939; Thompson, 1939). In general, it may be said that the physiological action of antigonadotrophic sera upon the hypophysis and gonads of normal animals has been shown to simulate simultaneously in a single animal both the effects of castration and hypophysectomy. Although at the present time, antigonadotrophic substances are believed to be closely allied to immune bodies (Zondek and Sulman, 1942), the mechanism of the inhibitory action has not been adequately explained. In speculating as to the probable manner in which inhibition of the endogenous gonadotrophic secretion is obtained by the antigonadotrophic serum, it is conceivable that the antiserum exercises its inhibitory effect by one of the following two *modi operandi*:

1. The antiserum might cause some alteration in the enzyme systems or other changes in the gonads so that they cannot respond to endogenous or administered gonadotrophic hormone.

2. The antiserum might act on the gonadotrophic hormone directly and either *a*, render it inactive *in situ* in the pituitary gland, or *b*, combine with the hormone in the blood after its release from the pituitary gland and thus inactivate it. However, upon consideration of these conceivable *modi operandi*, with respect to experimental findings previously reported, it is likely that the antihormone does not exert a direct effect upon the end organ (gonads), nor upon the gonadotrophic hormone in the hypophysis itself.

The possibility that the antigonadotrophic substance may act on the end organ, the gonads, to induce a generalized tissue refractoriness may be eliminated if we consider the work of other investigators on both the antigonadotrophic and anti-thyrotrophic substances. The experiments of Selye, Collip and Thompson (1934) showed that the gonads of rats refractory to one type of gonadotrophic preparation can still be made to respond to a gonad-stimulating hormone prepared from a different source. Similarly, Werner (1936 and 1938) has shown that the thyroid gland of guinea pigs rendered refractory to a crude thyrotrophic preparation of beef pituitary can be made to respond to a purified thyroid-

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stimulating extract, also made from beef hypophyses. In addition, Okkels, (1937) by use of the perfusion apparatus, demonstrated that the thyroid gland from an animal refractory to thyrotrophic stimulation as a result of the presence of antithyrotrophic hormone in the blood, will still respond to thyrotrophic hormone *in vitro*. These results indicate that there is no cytological immunity or tissue refractoriness in the end organ (*viz.*, thyroid or gonad) of an animal under the influence of antitrophic substances of the pituitary gland, and refute the concept of alteration of tissue reactivity as the mechanism of the inhibitory action of antihormones.

Elimination of the end organ as the possible locus of action of the antisera suggests that the neutralizing effect of the antigenadotrophic sera depends upon a direct action of the sera upon the secretion of the pituitary gonadotrophic hormone before or after its release from the gland. By determining the gonad-stimulating action of the pituitary glands of rats treated with antihormone for long periods of time, it has been shown that the antigenadotrophic serum does not inhibit or neutralize the gonad-stimulating hormone in the hypophyses of the injected rats (Meyer, Kupperman and Finerty, 1942). In fact, assay of the pituitary glands of these animals treated with antihormone showed their gonadotrophic content to exceed that of the normal gland and approximate the gonad-stimulating action of the hypophyses of castrated rats.

As the antigenadotrophic serum does not neutralize the gonad-stimulating hormone in the pituitary gland, it may affect its release or neutralize the hormone after its release from the hypophysis. In consequence, the following experiments, in which parabiotic rats were used, were planned to determine whether antigenadotrophic serum can neutralize the endogenous gonadotrophic hormone in the blood stream of the injected animal.

PROCEDURE. Eighteen 32 day old male rats were gonadectomized and each placed in parabiosis between two littermate females. The right female of ten of the parabiotic triplets was injected subcutaneously with 0.2 cc. of antigenadotrophic serum five hours after the operation, and the injections were continued every day for 10 days. The antihormone-injected triplets, together with the eight remaining untreated control parabionts, were all autopsied on the 42nd day of life. At autopsy the ovaries of the right and left female, and the seminal vesicles and prostate of the middle castrated partner were removed and weighed to the nearest milligram.

The antigenadotrophic serum used in these experiments was prepared by centrifugation of the blood obtained by cardiac puncture from rabbits repeatedly injected with an aqueous supercentrifuged extract of whole sheep pituitary glands. The unfractionated gonadotrophic extract was prepared according to the method of McShan and Meyer (1940). The serum was aspecific in nature since it inhibited the gonad-stimulating action of hog, human, rat and sheep pituitary extracts and gonadotrophins found in human pregnancy urine and pregnant mares' serum. In addition, the serum effectively inhibited the action of endogenous gonadotrophic hormones of the normal and gonadectomized rat (Kupperman, Meyer and Hertz, 1939; Meyer and Kupperman, 1939).

RESULTS AND DISCUSSION. The data presented in table 1 show that in the control parabionts the hypersecretion of the gonadotrophic hormone from the pituitary gland of the middle castrated male is equally effective in causing ovarian growth in both the left and right female. The average ovarian weight of the right and left female of the control triplets was 67 and 55 mgm., respectively. Administration of antigonadotrophic rabbit serum to one of the female

TABLE 1

The effect of antigonadotrophic sera upon ovarian hypertrophy in parabiotic triplets

OV. WT., LEFT FEMALE	MIDDLE CASTRATED MALE		OV. WT., RIGHT FEMALE
	S. V.†	Prostate	
Experimental*			
mgm.	mgm.	mgm.	mgm.
47	7	16	22
12	7	15	13
48	9	21	11
130	8	15	8
22	9	14	13
38	9	15	13
25	9	12	9
117	11	26	15
138	7	15	20
63	9	21	12
Average.....64 ±14.86	9.5 ±0.52	17 ±1.34	13.6 ±1.39
Controls			
64	9	20	56
24	10	23	41
69	12	26	68
42	10	16	117
12	14	29	13
70	9	23	109
124	6	21	103
40	7	18	34
Average.....55 ±12.29	9.6 ±0.82	22 ±1.5	67 ±13.68

* Right female treated with antigonadotrophic serum for ten days.

† Seminal vesicles.

parabionts (right), however, prevented ovarian hypertrophy from occurring in the injected partner only. The average ovarian weight of the right partner given antihormone was 13.6 mgm., as compared to 64 mgm. for that of the left untreated female.

These data on the parabiotic triplets adequately indicate that neutralization of the endogenous gonadotrophic hormone can take place in the blood stream of the injected animal. This concept is supported by the fact that inhibition of

the gonad-stimulating action of the pituitary gland of the middle castrated partner occurred in the injected rat only. The control series show that the two females parabiotically united to a castrated litter-mate male received sufficient gonadotrophic hormone in their blood from the hypophysis of the middle castrated partner to cause extensive hypertrophy of their ovaries. As the antiserum prevented gonadal hypertrophy in the injected animal with little effect upon the ovarian hypertrophy of the non-injected partner, the gonadotrophic hormone from the middle castrated male must have been mainly nullified in the blood stream of the injected animal. Furthermore, the data also show that little, if any, of the administered antigonadotrophic substance crossed from the injected animal to the castrated male, since the average ovarian weight of the untreated female was not significantly different from that of the comparable partner in the control parabionts.

In agreement with the data obtained from the triplets we have also shown in other experiments that antigonadotrophic serum inhibited the effectiveness of the endogenous gonadotrophic secretion of the hypophysis of a castrated rat in parabiosis with a normal female when the serum is injected into either the castrated or normal partner (Kupperman, Meyer and Hertz, 1939). In light of the fact that the data in the present paper indicate that little if any antigonadotrophic serum crosses over from one animal to another, the experimental findings in the parabiotic pairs suggest 1, that the administration of antiserum to the castrated partner of parabiotic rats prevented gonadal hypertrophy in the intact member by neutralizing the gonad-stimulating hormone in the blood stream of the castrated rat, and 2, that stimulation of the gonads by endogenous gonadotrophic secretion was effectively inhibited in the intact animal despite the fact that the antiserum was injected into the castrate partner. It is to be emphasized that gonadal hypertrophy was prevented in the intact animal notwithstanding the fact that the gonads were at no time under the influence of the antigonadotrophic serum.

In conclusion it may be said that by simultaneous consideration of the experimental findings in parabiotic triplets and the data from cognate work reported by this and other laboratories, it seems reasonable to suggest that the inhibitory action of antihormone is accomplished not by inactivating the gonadotrophic hormone in the hypophysis nor by rendering the gonads refractory to gonadotrophic stimulation but by neutralization of the gonadotrophic hormone, exogenous or endogenous, in the blood stream of the injected animal.

SUMMARY

A study of the mechanism of the inhibitory action of antigonadotrophic serum was made in parabiotic triplet rats composed of two females joined to a middle castrated male. In the control parabiotics it was found that the average ovarian hypertrophy observed in each of the two female rats united to the middle castrated male was comparable. Injections of antigonadotrophic serum into one female (the right) of the parabiotic triplets effectively prevented gonadal stimulation in the injected female only. Ovarian hypertrophy occurred

only in the uninjected female of the experimental triple parabionts, and approximated that seen in either of the females of the control triplets. It is concluded from these experiments that antigonadotrophic sera can inhibit the endogenous gonad-stimulating hormone in the blood stream after its release from the pituitary gland.

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ALTERATIONS IN THE PROPERTIES OF DOG HEPATIC BILE WITH INCREASING AGE OF THE CHRONIC BILIARY FISTULA

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During the course of certain studies on the effect of various drugs upon the 24 hour output and upon a number of physical and chemical properties of hepatic bile obtained from dogs by the Rous and McMaster (7) technique, changes were seen to occur in the appearance and properties of the bile as time went on. It was decided to investigate these changes more systematically and a series of estimations was performed upon hepatic bile from five dogs, of 10 to 15 kilos body weight, daily for a period of 15 weeks. In none of these animals was bile returned to the gastro-intestinal tract and all of them were dead shortly before or after 15 weeks of chronic biliary fistulous drainage. The dogs were given a diet of dog chow supplemented with table scraps, and were exercised daily outdoors. They became accustomed to the dressings holding the cage and rubber bag for collecting bile, offered no resistance to the daily removal of bile, and, until a few days before death, appeared quite healthy and active, although they steadily lost weight.

The following estimations were made daily. The 24 hour volume output of hepatic bile was noted, the specific gravity was determined and the relative viscosity estimated in the Ostwald viscosity pipette. Aliquots were dried at 90°C. for 100 hours and the total solids calculated as grams per 100 ml. of hepatic bile. The following chemical analyses were performed by adaptations of the methods of the authors referred to: sodium (5), potassium (4), chloride (10), inorganic phosphate (2), total fatty acids (1), total cholesterol (1), ester cholesterol (1), free cholesterol (1), bilirubin (9) and bile salts (6). When the data had been tabulated, it was found that the main changes occurred just after the first three weeks and during the last three weeks. Hence, to simplify presentation of the voluminous data, all results were averaged over periods of three weeks and these means have been collected in table 1.

The volume output of hepatic bile rose from a mean of 7.3 ml. per kilo per 24 hours in the first three weeks to a peak value of 13.2 ml. during the 10th to 12th weeks, after which it declined. The specific gravity, relative viscosity and per cent total solids steadily declined with increasing age of the biliary fistula. Coincidentally there was a marked decrease in the concentration of total fatty acids and lesser decreases in the concentration of total cholesterol, ester cholesterol, inorganic phosphate, bilirubin and bile salts. There was no consistent change in the concentration of free cholesterol while the levels of sodium, potassium and chloride rose throughout most of the experiment.

These changes in the chemistry of hepatic bile represent changes in concentration but since the volume output was also changing, a somewhat different,

and perhaps more significant, picture is obtained if the various components of hepatic bile listed in table 1 are calculated as the average daily output per kilo body weight rather than as concentration in bile. Thus it will be found that while the dogs continued to put out increasing volumes of hepatic bile of decreasing viscosity and specific gravity, the actual total daily output of solids remained fairly constant until after the 12th week when it declined. Further, while the average daily output of total solids remained fairly constant, the composition of the total solids changed markedly as time went on and these changes may, in general, be divided into three periods. These periods are

TABLE 1

Changes in the properties of dog hepatic bile with increasing age of the chronic biliary fistula

PROPERTY	AGE OF FISTULA IN WEEKS				
	1 to 3	4 to 6	7 to 9	10 to 12	13 to 15
24 hr. volume (ml. per kilo per 24 hours).....	7.3	8.3	11.8	13.2	9.3
Specific gravity.....	1.0089	1.0088	1.0051	1.0055	1.0047
Relative viscosity (dist. water = 1.000).....	1.351	1.193	1.171	1.118	1.086
Total solids (gram per 100 ml.).....	4.02	2.67	2.34	2.14	1.70
Total fatty acids (mgm. per 100 ml.).....	359	114	52	24	27
Total cholesterol (mgm. per 100 ml.).....	51	41	37	11	18
Ester cholesterol (mgm. per 100 ml.).....	33	22	25	3	5
Free cholesterol (mgm. per 100 ml.).....	18	19	12	8	13
Sodium (mgm. per 100 ml.)....	344	362	374	371	385
Potassium (mgm. per 100 ml.) ..	38	46	49	44	36
Chlorides (mgm. per 100 ml.)...	323	438	494	510	496
Bilirubin (mgm. per 100 ml.) ..	38	14	9	7	6
Bile salts (gram per 100 ml.)...	2.8	1.6	1.2	1.0	0.5
Inorganic phosphate (mgm. per 100 ml.).....	25	13	15	18	12

a, immediately after the first three weeks of biliary drainage; b, from the 4th to the 12th weeks of chronic biliary drainage, and c, after the 12th week.

The first period, occurring just after three weeks of fistulous drainage of hepatic bile, was characterized by a decrease in the output of total fatty acids, bilirubin and bile salts. The decreased output of total fatty acids, which included fatty acids from all saponifiable compounds in bile, averaged about 65 per cent, of bilirubin about 60 per cent and of bile salts about 35 per cent. To compensate for the lessened daily output of these substances in hepatic bile, there was an increase in the average daily output of chloride (about plus 55 per cent), potassium (about plus 35 per cent) and sodium (about 20 per cent), which may be taken to indicate daily increased outputs of sodium and potassium chlorides.

In the second period, from the 4th to the 12th weeks of fistulous drainage of hepatic bile, there occurred a gradual increase in the output of sodium, potassium and chloride. The sodium output increased to a mean maximal amount which was some 95 per cent greater than the initial output, potassium to a maximal increase of 107 per cent and chloride to a maximal increase of 187 per cent greater than the initial. These increases in the daily output of salt in bile were offset by a further decline in the output of total fatty acids. At the end of 12 weeks of fistulous drainage, the average daily output of total solids was exactly the same as it was at the beginning of the collection of bile but the composition of total solids had changed markedly, as noted above.

The third period, after 12 weeks of fistulous drainage of hepatic bile, was characterized by a decline in the volume output, a decline in the amount of total solids, chlorides, sodium, potassium, bilirubin, bile salts, total cholesterol and ester cholesterol. The daily output of sodium, potassium and chlorides, though below that of the previous period, was still somewhat higher than that at the beginning of the experiment. The output of total fatty acids was less than one-tenth the initial output, of total cholesterol less than one-half, of ester cholesterol about one-fifth, of bilirubin about one-fifth and of bile acids about one quarter. The sudden decline in the daily output of total cholesterol and ester cholesterol actually occurred a little earlier, between the 10th and 12th weeks. In this period, which preceded by some days the death of the dogs, the bile appeared thin and pale in color.

Throughout the fifteen weeks of the experiment there were no significant changes in the daily output of free cholesterol or inorganic phosphate. The daily average amount of these substances varied up and down but not consistently either way.

The results obtained permit certain definite conclusions and are conducive of much speculation regarding the physiology of biliary secretion in particular and its effect upon body metabolism in general. In the first place, the results herein obtained emphasize that the chemical and physical properties of hepatic bile vary greatly with the age of the biliary fistula. This factor should be taken into account, not only in studies upon the "normal" properties of bile, but also where various mechanisms, drugs, chemicals, etc., which affect biliary secretion are being investigated. Such has seldom been the case in the past. Previous accounts of the quantitative properties of bile have been notoriously variable and other factors responsible for this variation have been discussed in many reviews, for example that of Sobotka (8).

In the second place, it is well known that bile is essential to life. This statement is based upon the fact that animals with a continuous biliary fistula will eventually die unless bile is returned to the gastro-intestinal tract. The cause of death has been generally ascribed to deprivation of some constituent elaborated by the liver and peculiar to bile and essential to life (see, for example, Hawkins and Whipple (3)). Evidence obtained in the present investigation indicates that another and quite simple factor may be equally or more responsible for the death of these chronic biliary fistulous animals. This factor is the drainage

from the body of inorganic salts which, as shown herein, gradually increases with increasing age of the chronic fistula in dogs. In some animals, such as guinea pigs, total removal of bile results in death within a day or so (8). In guinea pigs, the volume of bile per day, relative to body weight, is much greater than in dogs or man, and it would seem quite plausible that the unchecked outflow of bile in guinea pigs causes a rapid and fatal loss of body salts. The production of bile bears some analogy to the production of urine, in that many substances are first discharged from the liver and then reabsorbed in the gall bladder or intestines, just as occurs, in somewhat like manner, in the glomeruli and tubules respectively of the kidneys. Thus the continuous removal from the body of hepatic bile might be compared to the continuous removal of the glomerular filtrate in the kidneys and it is obvious that under the latter circumstance animals could not survive for long.

SUMMARY

Daily estimations were made for a period of 15 weeks upon hepatic bile collected from five chronic biliary fistula dogs. The 24 hour volume output gradually rose until after the 12th week when it began to decline. The specific gravity and relative viscosity steadily declined throughout. The daily output of total solids remained fairly constant until toward the end of the experiment but the composition of the total solids underwent considerable change which could be divided into three periods. In the first period, immediately after some three weeks of fistulous drainage, there was a marked decrease in the output of total fatty acids, bilirubin and bile salts with an increase in the output of sodium, potassium and chlorides. In the second period, from the 4th to the 12th weeks of biliary drainage, there was a further gradual increase in the output of sodium, potassium and chlorides and a continued decline in the output of total fatty acids. In the third period, after the 12th week and just before the death of the animals, there was a decline in the volume output of bile and in the amount of total solids, sodium, potassium, chlorides, bilirubin, bile salts, total cholesterol and ester cholesterol. Throughout the experiment, there were no marked or consistent changes in the average daily output of inorganic phosphate or free cholesterol. The significance of these changes is discussed.

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AGE, SEX, CARBOHYDRATE, ADRENAL CORTEX AND OTHER FACTORS IN ANOXIA¹

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Investigations of anoxia have increased many-fold within the past few years, the curve of increase following somewhat the tempo of war. Experiments which were begun about four years ago in this laboratory have been reported only in part (Van Middlesworth *et al.*, 1944; Kline and Britton, 1945), and it would now seem opportune to record other phases of our results. Some correlative experiments by others are reviewed later.

METHODS. The work was carried out on rats, rabbits, cats, dogs, opossums and a few other species. A series of 18 barometric chambers, each of 9-liter capacity, was arranged for making tests on adult individuals, or in some cases two or more young animals were observed in each chamber. Ventilation was carried out at the rate of 2 liters per minute at S.T.P., and increased in inverse relation to decreased pressure, in the "ventilated chamber" (see Van Middlesworth *et al.*, 1944, for further details). In earlier experiments especially the air-current was reduced to small leakages—designated "closed chamber"—in which case the evacuating pump was tripped to maintain low pressure (thus providing ventilation) usually about 6 times per hour. Given the same pressure conditions, etc., survivals as might be expected were longer in the thoroughly ventilated chamber, in part because the closed type imposed some degree of hypercapnia. All our evidence indicated, however, that death was due primarily to hypoxia. Pressure in both chamber types was kept constant to within 5 mm. Hg. Extensive control tests were carried out.

Different low pressures were used as indicated in order to secure clearer end results as well as more practicable exposure periods in the different series. Two pressure levels—320 mm. Hg (an altitude equivalent of 22,000 ft.) and 162 mm. Hg (37,000 ft.)—were utilized for the closed and ventilated chamber tests respectively. In most experiments animals were removed from the chambers just at the point of death, and restoration was usually possible. The end-point criterion of survival was the very brief gasping period (10–30 sec.) which usually appeared one or two minutes after a short comatose-convulsive period. When two young or small animals were used in one chamber, the last respiratory effort was taken as the survival criterion. Only in cases of marked difference in survival time of experimental animals compared to controls were results considered positive.

The Folin-Malmros micromethod of blood sugar determination (1929) was used, combined with a modified Pflüger technique for glycogen analysis. Ad-

¹ Grateful acknowledgment is made of aid received in this investigation from the Endocrinology Fund of the National Research Council, 1941.

renalectomy was performed by the ventral approach under ether. In the case of very young rats the operation lasted usually no more than 2 minutes, and was observed to affect general activity little if at all. Laparotomy and other procedures except actual gland removal were performed on the controls (sham operation).

RESULTS. Age and anoxia. It appears from table 1 that fetal rats near term are able to withstand anoxic conditions about 10 times as well as adults, and newborn animals 5 to 10 times as well. A sharp drop in resistibility continues for about a week after birth; then the curve declines slowly, to fall actually below the adult type at 20 to 40 days (fig. 1). Not until some weeks later is the adult level of resistance found. Infant rats showed no sex differences in survival.

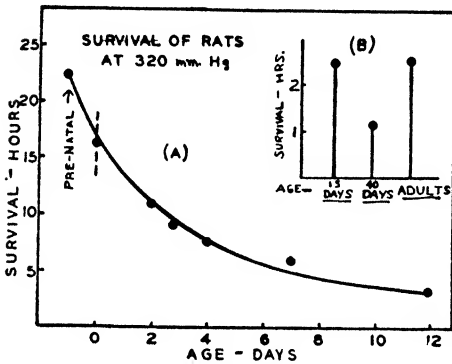


Fig. 1

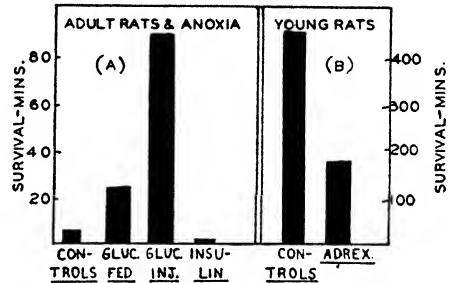


Fig. 3

Fig. 1. Showing high resistance of fetal rats to anoxia (A); that of newborn animals is less; and at 30-40 days of age resistance is actually lower than that of adults (B). "Closed" chamber. Each spot represents an average of 6 experiments.

Fig. 3. (A): Exposure of rats to 162 mm. Hg, ventilated chamber (see table 2). Note striking benefit of glucose. (B) Infant rats, 8 controls and 10 adrenalectomized, exposed to 162 mm. Hg, "closed" chamber (see text).

Young dogs, cats, rabbits and opossums were also observed to tolerate anoxia much better than older individuals (table 1). Young opossums of about 5 days' pouch age (equivalent to very young fetuses) were exceedingly resistant to low oxygen tension; 9 survived over 50 hours in the closed chamber at 320 mm. Hg. At about 15 days' pouch age the survival time was reduced to 20 hours (11 cases).

Species. Among the different animal species studied, the American brown bat (*Myotis subulatus*) showed extraordinary resistance (table 1). In some cases these animals lasted about 50 hours at 320 mm. Hg, and the group of 22 adults averaged 30 hours—much longer than any other adult mammal studied.

The adult opossum (*Didelphys virginiana*), a very primitive type, showed surprisingly low tolerance to reduced barometric pressure. Birds (duck, swift) resisted anoxia well.

Sex factors. Over the range of environmental temperatures examined—16° to 33°—female rats showed definitely superior performance over males. Nearly

TABLE 1

Survival of young and adult animals of different species at reduced barometric pressure

Survival to coma at 320 mm. Hg = 22,000 ft. alt. "Closed" chamber; temp. c. 26° (see text).

ANIMAL	AGE	NO. TESTED	RANGE OF SURVIVAL TIME	AV. SURVIVAL TIME
Rat.....	Fetal (near term)	7	20°30'–24°30'	22°30'
Rat.....	New born	6	12°00'–25°00'	16°30'
Rat.....	2 days	5	9°30'–11°00'	10°45'
Rat.....	3 days	5	7°30'– 9°00'	8°30'
Rat.....	4 days	7	7°25'– 8°40'	7°50'
Rat.....	7 days	10	5°30'– 6°30'	5°30'
Rat.....	12 days	6	3°00'– 4°00'	3°30'
Rat.....	15 days	8	1°55'– 2°53'	2°26'
Rat.....	20 days	6	1°05'– 1°30'	1°15'
Rat.....	40 days	4	0°50'– 1°00'	0°58'
Rat.....	Adult	50	2°00'– 3°30'	2°30'
Cat.....	3 days	2	9°00'– 9°30'	9°15'
Cat.....	16 days	6	3°00'– 5°20'	4°35'
Cat.....	4 weeks	4	2°10'– 2°25'	2°20'
Cat.....	9 weeks	6	0°25'– 0°35'	0°30'
Cat.....	Half-grown (small)	4	2°00'– 2°50'	2°20'
Dog.....	1 day	9	5°30'– 8°00'	6°20'
Dog.....	3 days	5	3°00'– 4°00'	3°30'
Rabbit.....	36 hours	10	11°30'–16°16'	12°50'
Rabbit.....	17 days	8	2°12'– 2°50'	2°25'
Rabbit.....	26 days	8	1°00'– 1°36'	1°51'
Rabbit.....	33 days	7	1°09'– 1°35'	1°22'
Opossum.....	Pouch (c. 5 days)	9	45°00'–55°00'	50°00'*
Opossum.....	Pouch (c. 15 days)	16	18°00'–23°00'	20°00'*
Opossum.....	Adult (small)	3	0°35'– 0°45'	40'
Bat.....	Adult	22	16°15'–52°00'	30°00'
Duck.....	2 days	8	5°00'– 6°45'	6°00'
Duck.....	3 days	4	4°45'– 6°45'	6°00'
Chimney-swift.....	Adult	2		8°00'

* Approximately; some died overnight.

2000 tests are represented in figure 2, carried out in the closed chamber at 320 mm. Hg. In a number of other experimental groups exposed in the ventilated chamber (282 cases), the results similarly favored markedly the female groups

(see table 2). A few animals were used two or three times, with rest periods of a week or so intervening.

At 17°, females survived on the average 260 minutes compared to 190 minutes for males, or 40 per cent longer; at 27° the advantage was about 20 per cent. While near approximation of the curves at higher temperatures was apparent,

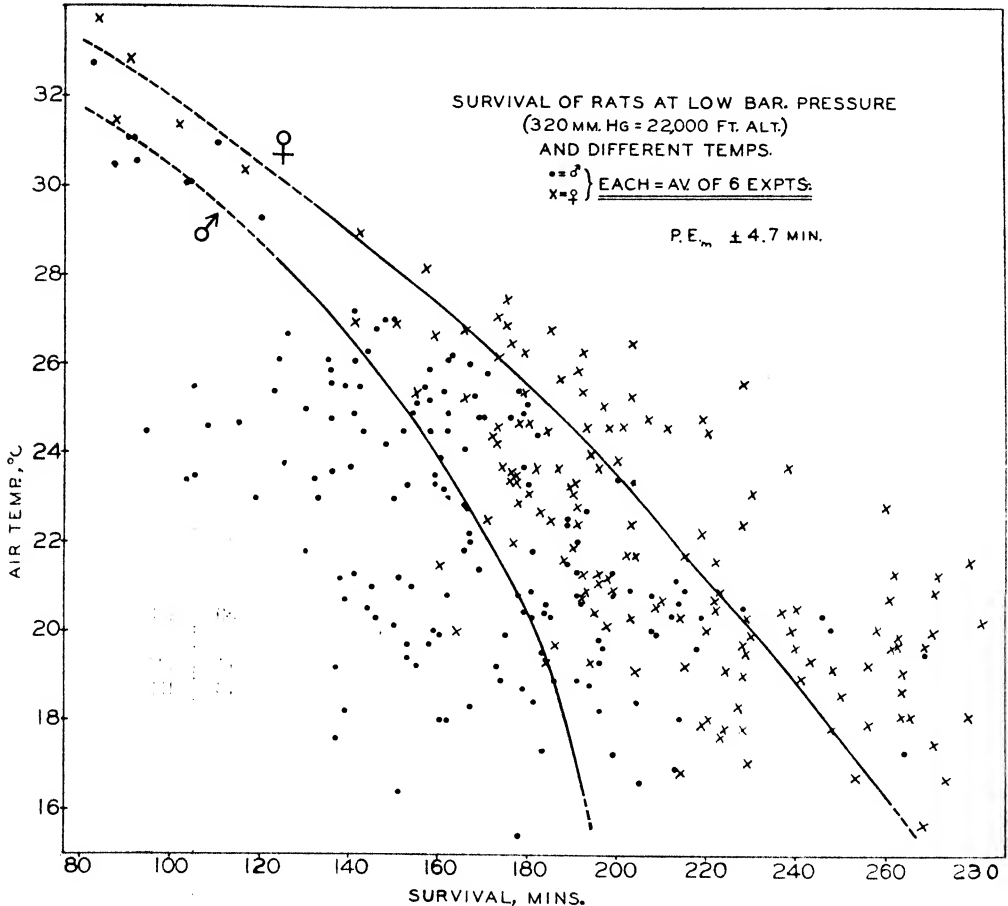


Fig. 2. Marked differences in male and female resistibility to anoxia are apparent. About 2000 separate tests are represented in the graph.

differences were present still at 32° to 33°. Nearly all the animals used were young adults, and weighed between 150 and 200 grams. The finding of sex differences was made first by chance, and the rather large series of experiments on this phase of the work accumulated during the course of other related tests.

In the ventilated chamber at the severely low pressure of 162 mm. Hg, it may be noted, female survivals were sometimes 50 to over 100 per cent longer

TABLE 2

Survival of rats on exposure to 162 mm. Hg bar. pressure (eq. 37,000 ft. alt.) under different test conditions

Ventilated chamber; Temp. c. 26°. See text for further details.

NO. RATS TESTED	SEX	TREATMENT	AVERAGE SURVIVAL TIME	BLOOD SUGAR		
				Initial	After treat- ment	After anoxia
(A) CO ₂ O ₂ (15/85) and other gases; glucose. All fasted 18 hours						
22	F	Controls	8	72		70
20	M	Controls	7	75		70
4	M	O ₂ for 60 min.	9			
4	M	CO ₂ 1 min.	7			
6	F	CO ₂ /O ₂ 60 min.	6	75	92	72
6	M	CO ₂ /O ₂ 60 min.	7	77	96	72
4	F	CO ₂ /O ₂ 90 min.	13	84	113	69
4	M	CO ₂ /O ₂ 90 min.	8	85	102	70
4	F	CO ₂ /O ₂ 120 min.	20	81	98	66
4	M	CO ₂ /O ₂ 120 min.	12	83	93	67
4	F	Carb.-fed, 7 days	15	80		72
7	F	Glucose inj., 7 days	88	79	132	105
8	M	Glucose inj., 7 days	32	80	130	102
13	F	Glucose 1 week + CO ₂ /O ₂ 60 min.	125	71	141	139
10	M	Glucose 1 week + CO ₂ /O ₂ 60 min.	78	73	139	137
(B) CO ₂ /O ₂ (15/85) gas mixture. Fully-fed rats						
10	F	Controls	10	93		91
14	M	Controls	8	100		93
16	F	CO ₂ /O ₂ for 30 min.	17			
16	M	CO ₂ /O ₂ for 30 min.	11			
16	F	CO ₂ /O ₂ 60 min.	43	99	118	87
16	M	CO ₂ /O ₂ 60 min.	34	99	107	70
20	F	CO ₂ /O ₂ 90 min.	38			
20	M	CO ₂ /O ₂ 90 min.	16			
20	M	CO ₂ /O ₂ 120 min.	38	100	125	91
20	M	CO ₂ /O ₂ 120 min.	21	99	124	86

TABLE 2—*Concluded*

NO. RATS TESTED	SEX	TREATMENT	AVERAGE SURVIVAL TIME	BLOOD SUGAR		
				Initial	After treat- ment	After anoxia
(C) Adrenalin, insulin, pregnancy, estrus, etc.; 18 hours fasting						
4	F	Adrenalin	11	71	141	139
5	M	Adrenalin	6	73	139	137
12	M	Insulin, 1u./100 gm.	1	61		53
		Insulin, 0.5u./100 gm.,				
3	M	0 min. after injec.	7	71		63
3	M	15 min. after injec.	4	75		59
3	M	30 min. after injec.	1	60		45
5	F	Pregnant, near term	11			
11	F	In estrus	7	72		69
8	F	In diestrus	10	70		73
7	F	Oophorectomy	19	71		63
9	M	Castrated	8	74		64
8	M	Castrated and estrogen	11	73		68

than those of males. Sex differences in survival were especially evident in the glucose and carbon dioxide treated cases (table 2).

In a number of tests, the possible involvement of estrus and estrogens (stilbestrol), the gonads, and pregnancy was considered (table 2, C). One group only (oophorectomized rats) showed a significant increase in survival time, however—an average of 19 mins. compared to the control time of 8 minutes.

Environmental temperature. Differences in survival times on exposure to anoxia at different surrounding temperatures are readily evident from figure 2, and little amplification is necessary. The survival of female rats is increased 100 per cent when the chamber temperature is decreased by about 14°—e.g., at 30° the average time is 130 minutes, while at 16° a period of 260 minutes is observed. The inverse proportionality for males does not involve quite so steep a curve of change except at higher temperature levels. Considering both sexes, a 10° fall in temperature increased survival time about 50 per cent. It is found statistically that at low temperatures the P.E._m for both sexes is about ± 10 minutes; at moderate temperatures the range is from ± 28 to ± 5.7 minutes, while at higher values the P.E._m is uncertain because of fewer observations.

Carbon dioxide; oxygen. In a number of experimental series we made tests utilizing materials which might influence individual tolerance to reduced oxygen tensions. The low barometric pressure of 162 mm. Hg was used, since this constituted a critical level at which most untreated adult rats survived only a

few minutes; it was therefore a severe test of the effect of a possible ameliorative factor.

Administration of oxygen (mixed with a very small amount of air) for one hour before exposure to low barometric pressure did not improve resistance significantly. Pre-exposure to nearly pure CO_2 for 60 seconds also did not affect the performance of another series of rats (table 2, A).

Using a CO_2/O_2 pre-induction gas mixture in the proportion of 15/85, however, it was noted that much longer survivals than non-treated controls were possible, especially in the well-fed group (table 2, B). Breathing of the gas was allowed in a chamber before decompression, for various periods as indicated. In some cases the life-span under subsequent anoxia was lengthened 4 or 5 times. When CO_2/O_2 was administered as well as glucose to fasted animals, survivals were increased still more—actually up to 15 times as long as controls in one group.

Glucose administration. The remarkably beneficial effects of carbohydrates in enabling animals to withstand exposure to severely low barometric pressures became evident early in our experiments (1941). Best results did not appear, however, until treatment had been given over protracted periods. The animals listed in table 2, A—about 200-gram adults—were given 5 cc. of 5 per cent glucose solution intraperitoneally twice daily for a period of seven days before exposure. Compared to untreated controls, such glucose-treated rats survived in some cases (females) more than ten times as long. When given CO_2/O_2 to breathe at the end of the glucose treatment period, survival times were extended 15-fold, i.e., from a brief 8-minute survival period (in 22 cases) to an average of 125 minutes (13 cases). Increments in male survival periods under glucose were also striking, although not so large as in the female groups.

A group of 4 female rats which were fed a rich carbohydrate diet for seven days—whole wheat, bread, glucose in milk—also withstood reduced oxygen levels better, showing an average of 15 minutes survival time compared to 8 minutes for fasted controls.

Convulsions, which usually occurred under anoxia in control cases, were never shown by the glucose-injected animals. Blood sugar levels were higher in the carbohydrate-treated group, and appeared to be correlated with longer survival periods. Results may be compared by reference to table 2.

Insulin. In 12 cases in which insulin was administered, severe reduction of survival time to 1 minute was observed (see table 2, C). Three rats were tested under anoxia 30 minutes after insulin was given, 3 at 60 minutes, and 6 at 75 minutes.

In 9 further cases, 3 exposed immediately after insulin had been given survived an average of 7 minutes, 3 tested 15 minutes later lasted 4 minutes, and 3 tested 30 minutes after injection lived only 1 minute in the low barometric chambers. Blood-sugar concentration after exposure showed a significant direct correlation with survival times, average levels of 63, 59 and 45 mgm. per cent being recorded in the above series respectively. In all these cases, severe convulsive seizures were observed.

Adrenal glands and hormones. A large amount of our experimental evidence from many different angles indicates involvement of the adrenals in anoxic contingencies. In a group of rats from which the adrenal glands had been removed, the following results compared to controls were observed:

Rats: Exposure to 320 mm. Hg (eq. 22,000 ft. alt.); closed chamber

	SURVIVAL TO COLLAPSE					
	At 22°	No. cases	At 24°	No. cases	At 26°	No. cases
	<i>min.</i>		<i>min.</i>		<i>min.</i>	
Adrex.....	110	8	121	8	82	9
Controls.....	173	25	160	25	141	25

Observations were made within the first two weeks after operation, during which time salt solutions were allowed in the diet. Animals which lived beyond this period began to show anoxia-survival times which approximated those of normal rats, a fact probably referable to accessory adreno-cortical growth.

Earlier experience which we had had with infant rats under anoxia suggested their use in adrenalectomy tests. Ease of handling such young individuals in small chambers, and the possible implication of the adrenal mechanism in off-setting anoxic difficulties, emphasized this idea. Rats 6 or 7 days old were given a small amount of ether and both adrenals were removed two hours or so before exposure to low barometric pressures.

Preliminarily, 2 adrenalectomized 6-day-old rats were tested with 2 litter-mate controls exposed together to 162 mm. Hg shortly after operation. Both the former animals succumbed quickly—one in 5 minutes and the other in 12 minutes—while the control pair remained in very good condition. The controls were returned to their mother.

In another series of 7-day-old rats, 4 adrenalectomized individuals were compared with 4 sham-operated litter-mates on exposure to 320 mm. Hg, chamber temperature 27°. In the former adrenalless group, survivals to death averaged 3 hours 50 minutes, and the control cases 6 hours 45 minutes.

Two other 6-day-old litters were utilized at the same barometric pressure (320 mm.) but a higher chamber temperature of 29°, with the following results:

Litter A—4 adrex.....	Average survival to death: 1 hr. 22 min.
2 controls.....	Average survival to death: 8 hr. 0 min.
Litter B—2 adrex.....	Average survival to death: 1 hr. 27 min.
2 controls.....	Average survival to death: 7 hr. 54 min.

At this higher temperature it will be observed that adrenalectomized rats tolerated anoxia very poorly, while sham-operated controls were still highly resistant.

Four adult male rats which were unilaterally adrenalectomized (left gland removed) were exposed daily to collapse to 320 mm. Hg over a period of 9 days, and the remaining right gland then excised and weighed. Seven controls were

similarly operated but not exposed to anoxia, and also examined after the 9-day interval. Results were as follows:

Male rats; (left adrenal gland out 9 days).

4 exposed cases.....	Right adrenal, weight aver.: 0.037% body weight
7 unexposed cases.....	Right adrenal, weight aver.: 0.021% body weight

The normal adrenal hypertrophy following unilateral excision was thus augmented under the stress of reduced oxygen levels. Liver weights were also found to be greater in the exposed series—an average of 3.02 grams (4 cases) compared to 2.48 grams in the controls (7 cases).

Suggesting adrenal involvement also are data on liver glycogen changes in adrenalectomized adult rats under anoxia. After recovering well from the operation, animals were exposed to 320 mm. Hg to the point of collapse, then sacrificed and compared with controls similarly exposed. Results were as follows:

	<i>Liver glycogen</i>
Adrenalectomized.....	5 cases, range 0.13–0.27 per cent, average 0.21 per cent
Controls.....	8 cases, range 1.12–2.21 per cent, average 1.83 per cent

Besides the very low liver glycogen levels shown above, blood sugar and heart glycogen values were also strikingly below normal in the adrenalectomized series.

In a further series, rabbits about one-third grown were tested before and after treatment with cortico-adrenal extract. All exposures were made at 320 mm. Hg. In 29 tests without extract, average survival time to collapse was 73 minutes, and the range 52 to 90 minutes. After extract treatment in 18 cases the average was 88 minutes, range 55 to 165 minutes. In 7 tests on rabbits injected with desoxycorticosterone, no significant difference in survival time compared to controls was noted. Three groups of rats (8, 8, 7–23 in all), also treated with D.C.A. over periods up to one week, similarly showed no difference in response compared to controls and to pre-treatment survival times.

A series of 10 rats treated with adrenalin (0.5 mgm. in oil per 100 grams rat, s.c.—table 2, C) showed practically unimproved performance compared to controls.

Serum electrolytes. Serum potassium, sodium and chloride levels of rats exposed to a barometric pressure of 320 mm. Hg to collapse were not significantly altered from the normal.

DISCUSSION. Many substances have been said to influence resistance to anoxia, including various foodstuffs and vitamins. Campbell (1939) concluded that certain proteins were beneficial, and meals of high protein value were considered important by King *et al.* (1945). The latter workers stated that carbohydrates, however, afforded a distinct advantage to fliers on the basis of visual and other tests. A number have reported that a rich carrot diet (Campbell, 1938; Nelson *et al.*, 1943; Hiestand and Miller, 1944) or minute amounts of riboflavin (Dietrich and Pendl, 1937) are helpful. Tested by other workers, vitamins were not found of utility (Hailman, 1944; see also Smith *et al.*, 1944).

It would seem more likely to us that protein, on the basis of its stimulation of heat output, would lower rather than raise one's resistance. In the present experiments, as later developed, carbohydrate substances appeared of outstanding benefit.

Young animals have been observed to tolerate various anoxic emergencies much better than adults (Reiss and Haurowitz, 1929; Avery and Johlin, 1932; Kabat, 1940; Cameron, 1941; Fazekas *et al.*, 1941). Carbon monoxide, nitrogen and other gases have been widely employed, and the isolated head of young animals has been used in many tests (Selle, 1944; Kabat and Dennis, 1939). Himwich and his collaborators have recently contributed many important papers in this field.

A suggestion has been made that relatively low oxygen demands of the central nervous system of young individuals may account for their high degree of resistance (Kabat and Dennis, 1939; Selle, 1944). It would hardly seem likely, however, that one protective factor only would be responsible for the phenomenon. Many mechanisms in the body, surely, work together to combat oxygen deficiency, with which condition (to some extent) the tissues of active individuals are confronted daily.

The relatively large size of the liver and its high concentration of glycogen (6-8 per cent) in young rats (Corey, 1935) would for example appear to be involved in the striking degree of tolerance observed; this is particularly so, in view of data on the importance of carbohydrates in anoxia. When liver glycogen levels have declined to approximately adult values, at 3 to 4 weeks of age (Britton and Silvette, 1932), anoxic resistance significantly reaches adult or even sub-adult levels.

Another fortifying element in connection with the resistance of fetal and young animals to anoxia might be found in the blood, with its peculiar form of hemoglobin (see Windle, 1940). Such fetal hemoglobin, stated to take up oxygen with great avidity, would be of considerable utility in those (temporary) asphyxial contingencies which are experienced rather often in fetal and early post-natal life.

From the present results it would seem pertinent to add also one hormonal factor, that supplied by the adrenal cortex. The large size of the adrenal glands—mostly cortex—compared to body weight during fetal life, and their rapid relative regression post-natally, go hand-in-hand with the experimental data on survival times submitted herein. Young male and female rats, in contrast to adults, showed equal resistance to anoxia—in keeping also with the lack of sex difference in adrenal weights in young animals. The lower resistance of 20 to 40-day-old rats may be referable to the marked speeding up of bodily activities on their becoming independent at weaning.

That rats may tolerate anoxic conditions better after thyroid removal or thiourea injection, while thyroxin administration may lower tolerance, has been reported by Leblond (1944). General metabolic activity may not be so important a factor, however, as some workers suggest. The primitive and torpid adult opossum tolerated low barometric pressures poorly in our experi-

ments, while the exceedingly active bat withstood reduced atmospheric pressures best of all the adult forms examined. It should be mentioned that all tests on bats were made outside of the usual hibernating season. Rather wide variations in individual responses of animals of the same species should, however, be noted (see range of survival time, table 1).

The striking sex differences in survival which we have observed are hardly explicable on a metabolic basis alone. Involvement of the sex glands specifically was not apparent in several series of tests. Possibly the larger relative size of adrenal glands in females, due mainly to the cortex, may here also have a part in the longer survival periods observed.

The effects of CO_2/O_2 breathing in prolonging survival of rats under anoxia may also not be explained by general metabolic (depression) changes only. No difference could be observed in the general activity of such treated animals. It is our belief that the results reported are more particularly referable to a sort of priming action, or a quick acclimatization process, which breathing of the gas mixture might afford. In this conception, of course, one would include the improved vascularization of important nerve centers which carbon dioxide in excess would tend to bring about. In the hyperpneic phase with its possible serious loss of CO_2 at the beginning of anoxic exposure, the organism would also be protected by pre-breathing CO_2 in high concentration. A rather advantageous shift in the hemoglobin oxygen-dissociation curve would also appear to be involved. Garasenko (1944) has observed, it may be noted, that CO_2/O_2 16/84 mixtures are beneficial at altitude equivalents up to 13,000 meters (465 mm. Hg).

By far the longest survivals were observed after animals had been given carbohydrates over a period of several days, and then exposed to reduced barometric pressures. Surviving in some cases nearly two hours at the high altitude equivalent of 37,000 ft., glucose-treated rats appeared in fairly good condition until the last few minutes; they suffered no convulsive seizures at the end, and recovered well on recompression to 760 mm. Hg.

Himwich and his co-workers (1942) have noted the effects of glucose and insulin on the survival of animals in an atmosphere of nitrogen. Further, they conclude that carbohydrate materials supply energy for cerebral activities during anoxia (Fazekas and Himwich, 1943). The action of insulin in decreasing and of cortico-adrenal extracts in increasing resistance appears significant in connection with these and other phases of the anoxia picture. Possibly the insulin effect is explicable on the basis of the rapidly-induced hypoglycemia. In this connection the hypoglycemic condition which generally follows the hyperglycemic phase in anoxia results in depletion of cardiac glycogen, and eventually dissolution sets in. The now well-recognized (but not fully understood) carbohydrate functions of the adrenal cortex (see Britton, 1930; Britton and Silvette, 1931) may thus in the anoxic status express themselves in better mobilization and utilization of glycogen reserves. Hence more glucose would eventually be made available to and usable by the harder-working heart and other continuously-laboring muscles (e.g., respiratory), as well as the central nervous system.

A considerable burden of evidence brought forward here points significantly, it is felt, to such important issues of the problem. The practical utility of several phases of the results is apparent. A review of others' work and the present results appear to justify the following tabulation:

Resistance to anoxia

<i>Relatively low</i>	<i>Relatively high</i>
Adult animals	Fetuses and infants
Males	Females
High air temperatures	Low temperatures
High metabolism	Low metabolism
Thyroxin injection	Thiourea or thyroidectomy
Hyperinsulinism	Glucose treatment
(Protein diet?)	Carbohydrate feeding
Adrenalectomy	Cortical extract injection
	CO ₂ /O ₂ pre-breathing

SUMMARY

Age, sex, species and environmental temperature are important factors in resistance to low barometric pressures.

Rat fetuses and immature pouch opossums are highly resistant to anoxia. Infant animals show sharp declines in resistibility over the first one or two weeks of life, then become actually less tolerant for a period than older or adult animals (cat, rat).

The relatively large size of the adrenal glands may be a factor in the superior resistance of young animals. Correlated probably are the high liver glycogen levels in infant rats. When glycogen levels have declined at 3 to 4 weeks of age, and the adrenals have also regressed (relative to rat weight), resistance to anoxia is found reduced even below that of adults.

Out of 6 different mammalian types studied, the American brown bat showed phenomenally high resistance to anoxia.

In nearly 2000 tests, adult female rats showed much longer anoxic survivals than males over the temperature range 16° to 33°. In 282 other cases (11 series), females were similarly predominant. The large size of the adrenal glands in females, due chiefly to cortical tissue, may be a factor in these results.

In infant male and female rats, in which adrenal weights are approximately the same, no sex differences in tolerance were observed.

Survival times under anoxia are inversely related to environmental temperature. For a 10° drop, survivals were increased about 50 per cent.

Glucose administration improves greatly the resistance of rats to low barometric pressures. Carbohydrate meals and especially glucose injections were effective. Fully-fed animals did somewhat better, also, than fasted controls.

Insulin markedly reduced the ability to withstand anoxia.

CO₂/O₂ (15/85) breathing before exposure to anoxic conditions was particularly helpful to fully-fed rats.

In combination, glucose injection and CO₂/O₂ pre-breathing proved most useful in raising resistance.

CO₂ and O₂ tested separately were not significantly effective as pre-exposure measures.

Serum potassium, sodium and chloride levels were not affected by anoxic exposure.

Beside the above data, a considerable array of evidence on young and adult animal responses point to specific adrenal involvement in anoxia. Infant rats without adrenals were strikingly reduced in resistibility, to levels indeed below those of normal adult animals. Experiments on adrenal hypertrophy and cortical extracts offered supporting evidence. Altogether the results strongly indicate that while other factors are involved, the adrenal cortex aids greatly in extending life under anoxic contingencies. This is probably effected especially through its carbohydrate-regulating mechanisms.

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THE SURVIVAL OF NON-ADRENALECTOMIZED RATS IN BURN SHOCK WITH AND WITHOUT ADRENAL CORTICAL HORMONE TREATMENT

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In studies from this laboratory (1, 2) it was shown that beef adrenal extracts and certain adrenal cortical steroids were without effect upon the resistance of non-adrenalectomized rats to limb ligation shock but that the administration of large doses of hog adrenal extracts to non-adrenalectomized rats raised their resistance to peptone shock.

In the present study burn shock was induced in non-adrenalectomized rats. A comparison was made of the incidence of survival among groups of rats which were treated with hog adrenal extracts, 11-desoxycorticosterone acetate, and control solutions. The results were negative.

METHODS. Normal male rats of the Sprague-Dawley strain, weighing 190 to 205 grams, were used. A short period of anesthesia was induced by the intraperitoneal injection of 18 mgm. of cyclopentenyl-allyl-barbituric acid sodium with added ether to obtain complete anesthesia during the burn. A rubber band was looped around the two back limbs at the level of the lower tibia and around the upper body just under the fore-limbs. The head, feet and tail were held together with fingers of one hand and the body of the animal was immersed to the level of the axilla and the base of the tail in the water of a constant temperature bath for 60 seconds. The temperatures used were 68°C. and 72°C. The extremities were excluded from the burn because those areas which are unprotected by hair are burned more severely and are chewed by the animal after recovery from the anesthetic. Following the burn each animal was kept without food or water in a separate compartment of a partitioned cage.

Extracts from hog adrenal glands were made up in sesame oil solution so that each dose was contained in 0.5 cc. All traces of epinephrine had been removed from these preparations. A control solution was prepared by dissolving physiologically inert material from adrenal extract in sesame oil so that it could not be distinguished from the active extract on the basis of appearance. The compound, 11-desoxycorticosterone acetate was purchased on the market (Schering) and had a concentration of 5.0 mgm. per cc. of oil. A control solution of oil was used.

All of the test solutions were submitted to the experimenter as "unknowns". The selection and matching of the rats with the test substances was by the following procedure. Following the burning of the rats a card was drawn at random from a thoroughly shuffled pack and the matching of the rats with the test solutions was made according to the designations on the card.

EXPERIMENTS AND RESULTS. The test solutions were administered immediately following the burn and again six, twenty-four and thirty hours later in those animals which survived. The incidence of survival at six, twenty-one, twenty-four, thirty, forty-five and forty-eight hours following the burn was the criterion used to judge the efficacy of the test substances.

TABLE 1

Number of rats surviving burn shock with and without treatment with hog adrenal extract

TEST SOLUTIONS	DOSE, HOG GLAND EQUIVALENT	DEGREES C	HOURS FOLLOWING BURN						
			0	6	21	24	30	45	48
231A	50 grams	72	50	50	32	26	21	17	16
231B	control	72	50	50	28	24	21	18	17
81206-1	50 grams	72	50	49	27	25	19	16	16
81206-2	control	72	50	50	23	20	16	16	14
270-1	control	72	50	50	25	22	18	15	15
270-2	100 grams	72	50	50	29	24	18	18	18
269-1	100 grams	68	50	49	44	44	40	37	34
269-2	control	68	50	50	46	44	43	37	37

TABLE 2

Number of rats surviving burn shock with and without treatment with 11-desoxycorticosterone acetate

TEST SOLUTION	DOSE	DEGREES C	HOURS FOLLOWING BURN						
			0	6	21	24	30	45	48
269-3	1.25 mgm.	72	50	50	29	24	18	12	12
269-4	control	72	50	50	34	29	22	19	19
269-5	control	72	50	48	28	24	19	16	16
269-6	2.50 mgm.	72	50	49	26	22	16	12	11
268-A-10	2.50 mgm.	68	50	49	38	38	37	34	32
268-B-10	control	68	50	49	34	34	33	31	31

The data on dosage, temperature and incidence of survival of rats treated with the cortical hormones and control solutions are summarized in tables 1 and 2. The administration of hog adrenal extract and of 11-desoxycorticosterone acetate was ineffective in increasing the incidence of survival.

DISCUSSION. Studies of the effect of adrenal cortical extracts and steroids on the resistance of animals and patients to stress have been reviewed by Swingle and Remington (3) and by Bergman et al. (4). There has been no satisfactory evidence that adrenal cortical hormones will increase the resistance of animals or patients having undamaged adrenal glands to any naturally occurring type

of shock. A number of problems remain for careful investigation. Thorn et al. (5) have recently reported that the pretreatment of non-adrenalectomized rats with massive doses of beef adrenal extracts increased their tolerance to low atmospheric pressures. Their methods of treatment deserve testing in non-adrenalectomized animals which have been subjected to other forms of stress.

SUMMARY

Normal male rats of 190 to 205 grams' weight were caused to develop burn shock by immersing them in water for 60 seconds at temperatures of either 68°C. or 72°C. A comparison was made of the incidence of survival up to 48 hours of rats which were treated with hog adrenal extracts, 11-desoxycorticosterone acetate and control solutions. The results were negative.

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THE CHANGES IN COMPONENTS A AND B OF PROTHROMBIN IN THE DOG FOLLOWING HEPATECTOMY¹

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It has been demonstrated (1, 2) that following hepatectomy the plasma prothrombin of the dog decreases, at first precipitously, and then at a slower rate until the death of the animal. This observation has been interpreted as evidence that the liver is concerned in the formation of prothrombin. The initial precipitous fall is probably due to the extensive manipulation of the liver involved in the operative procedure, since Lord (3) has shown that such manipulation causes a decrease of prothrombin to as low as 60 per cent of the value before the trauma.

Quick (4) has shown that prothrombin is composed of two separable components, which he designates as A and B. Component A disappears from oxalated plasma on storage, while component B disappears from the plasma of animals following administration of large doses of dicumarol.

With these observations in mind, we considered it of interest to study the changes in prothrombin of hepatectomized dogs, with particular attention to the changes in each of the components. We felt that such a study would indicate whether the liver was involved in the formation of both, or only one, of the components described by Quick. At the same time observations were made of the levels of the plasma total protein and fibrinogen, the hemoglobin, and the hematocrit. These observations are not directly connected with the main object of this investigation, but were obtained with the object of following the general condition of the dogs after hepatectomy. Since, however, these data present some points of interest they are included in this paper.

METHODS. *Hepatectomy.* Dogs ranging in weight from 7 to 15.5 kilos were hepatectomized by the one stage method of Firor and Stinson (5) as modified by Markowitz (6). Each dog was given morphine sulfate, 5 mgm. per kilo, approximately 30 minutes preoperatively. The operation was performed under open drop ether anesthesia. The hepatectomy was considered to be physiologically established at the time the portal vein was occluded and all times were calculated from this point. Following the operation glucose was supplied to the dog by continuous intravenous infusion at the rate of 0.5 gram per kilo per hour. The glucose was given as either a 5 per cent or 10 per cent solution, depending on the condition of the dog as shown by hematocrit and hemoglobin determinations.

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Blood samples of 4.5 cc. mixed with 0.5 cc. of 0.1 *M* sodium oxalate were collected for study before the operation and postoperatively at one hour intervals for the first four hours and at two hour intervals thereafter. On each of these samples the following determinations were made: 1, plasma prothrombin time; 2, plasma total protein; 3, plasma fibrinogen; 4, the hematocrit, and 5, the hemoglobin.

Prothrombin determinations. Plasma containing only prothrombin A was obtained by poisoning a rabbit with dicumarol (4). A daily dose of 10 mgm. of dicumarol per kilo was given by stomach tube for about 7 days, at which time the rabbit was bled by heart puncture. The blood was mixed with $\frac{1}{4}$ of its volume of 0.1 *M* sodium oxalate and the plasma obtained by centrifuging. This plasma had a prothrombin time exceeding ten minutes.

Considerable difficulty was encountered in preparing a plasma containing only component B. A report by one of us (F. L. M.) on the preparation of such a plasma will be published shortly and the details will, therefore, not be given here. Suitable preparations were obtained having prothrombin times of 150 to 300 seconds, corresponding to a prothrombin concentration of less than 1 per cent. A mixture of equal parts of the preparations gave prothrombin times of 15 to 20 seconds.

While the terms are admittedly inaccurate, for the sake of brevity the plasma preparations containing component A but deficient in B, and containing B but deficient in A will henceforth be called prothrombin A and prothrombin B respectively.

Prothrombin determinations were made by Quick's method (7) using thromboplastin prepared as described by Link (8). Since it was expected that the fibrinogen would be low in some of the samples, 0.1 cc. of fibrinogen, prepared by phosphate precipitation (9), was added to each determination. Triplicate prothrombin times were obtained for each sample unless these failed to check, in which case further determinations were made. For each dog a series of dilutions of the preoperative plasma with 0.15 *M* sodium chloride were prepared and the prothrombin times determined. The curve given by these dilutions was used in obtaining the relative prothrombin concentrations of the postoperative samples. The prothrombin time of each of the postoperative samples was determined on the undiluted plasma and on a 50 per cent dilution. The value for the 50 per cent dilution served as a basis of comparison for the prothrombin times obtained for the mixtures of the plasma with prothrombin A or B.

In determining the effect of the addition of prothrombin A and B on the prothrombin times an aliquot of each sample was mixed with an equal volume of prothrombin A and another aliquot with prothrombin B. The prothrombin time of each of these mixtures was then determined as described.

Other analyses. Total protein was calculated from the total nitrogen determined in 0.05 cc. of the plasma by the micro Kjeldahl procedure followed by Nesslerization. Fibrinogen was determined by the addition of thrombin to a suitable aliquot of the plasma, removing the clot, washing and drying it, and determining the nitrogen.

All the values for prothrombin, total protein and fibrinogen were corrected for dilution due to the addition of oxalate.

RESULTS. Successful operations were performed on five dogs with survival times from 3 to 10 hours. The data obtained on these dogs are given in table 1,

TABLE 1

The effect of hepatectomy on prothrombin, plasma protein, fibrinogen and hemoglobin concentrations

DOG AND SAMPLE NO.	HOURS POST- OPERA- TIVE	PROTHROMBIN VALUES					TOTAL PROTEIN	FIBRINO- GEN	HEMO- GLOBIN
		Undiluted		Diluted 50% with					
		Sec.	Per cent of pre- operative value	0.15M NaCl	Prothrom- bin A	Prothrom- bin B			
				sec.	sec.	sec.	gm./100 cc.	gm./100 cc.	gm./100 cc.
I-1		9.7	100	9.4	9.3	9.6	7.2		15.8
I-2	1½	10.8	39	11.6	10.4	11.2	5.6		14.6
I-3	2½	11.6	33	13.6	11.4	12.5	4.0	0.43	11.8
I-4	3½	13.4	22	15.2	13.2	13.8	3.0	0.33	9.9
I-5	4½	14.1	19	18.7	14.2	15.2	3.0	0.16	7.4
IV-1		9.7	100	11.2	10.2	9.7	6.0	0.28	15.0
IV-2	1½	10.3	52		11.0	11.0	4.1		14.7
IV-3	2½	11.6	37		11.4	12.4	4.0		13.8
IV-4	3	13.5	27	17.3	14.0	14.3	4.1	0.18	13.1
VI-1		9.6	100		9.1	9.1	8.2	0.49	18.0
VI-2	½	10.6	40	10.8	10.1	10.9	8.4	0.31	17.8
VI-3	2	13.6	24	15.5	10.9	15.3	5.8	0.26	16.2
VI-4	3½	19.7	13	26.6	13.1	22.6	3.7	0.12	12.4
VIII-1		10.3	100	12.7	12.6	10.8	5.7	0.31	16.9
VIII-2	1½	14.6	33	18.4	15.9	13.4	4.5	0.20	15.7
VIII-3	2	16.7	29	17.7	16.3	14.0	4.6	0.21	16.7
VIII-4	3½	17.6	27	21.9	17.7	15.8	3.8	0.17	14.8
VIII-5	4	18.3	26	24.2	17.5	16.5	3.6	0.15	14.4
VIII-6	6	20.6	23	26.6	21.0	17.3	3.2	0.13	13.9
VIII-7	8½	29.0	15	38.5	26.8	22.8	2.8	0.10	13.3
VIII-8	9½	43.5	10	65.0	30.8	37.5	2.6	0.04	12.5
IX-1		12.0	100	13.3	13.2	12.0	6.8	0.68	18.3
IX-2	1	17.4	25	19.2	17.6	14.9	5.2	0.48	18.5
IX-3	2	19.8	22	21.6	18.7	16.7	5.2	0.44	17.8
IX-4	3	21.8	19	25.1	20.6	18.4	5.1	0.42	17.4
IX-5	4½	23.5	18	27.0	20.2	19.2	4.6	0.42	16.9
IX-6	5½	29.5	13	36.5	24.0	23.1	3.5	0.27	14.6

and the prothrombin values are presented graphically in figure 1. The hemoglobin values are included in the table because they give an indication of the clinical condition of the dogs during the survival period. Dog I, for example, developed a hemorrhage after the operation was completed, and, in this case, death must be ascribed to the hemorrhage rather than to the hepatectomy.

Dog IV was in good condition throughout the survival period and death may be safely ascribed to the hepatectomy. Dog VI developed a hemorrhage due to leakage between the cannula and the vena cava and also showed postmortem indications of shock. Dog VIII showed signs of shock, as indicated clinically and by the hematocrit and hemoglobin values, at about the second hour postoperatively, but this was averted by increasing the supply of intravenous glucose. Dog IX showed signs of hemoconcentration between the second and fourth hour postoperatively, and while additional fluid was given failed to recover.

In spite of the variability of the clinical course followed by the various dogs there is a remarkable consistency in the changes in the prothrombin concentration. In each dog the prothrombin dropped within the first hour to between 60 per cent and 25 per cent of the preoperative concentration. This was followed by a continued but much slower drop to as low as 10 per cent during the

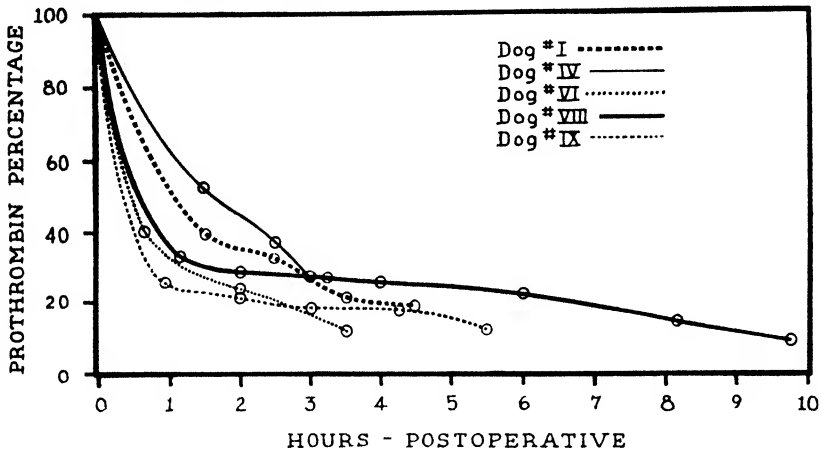


Fig. 1. The changes in total prothrombin in the dog following hepatectomy.

survival period. We are inclined to ascribe the initial sharp drop to the manipulation of the liver during the operation combined with the absence of the liver. The later slower fall is ascribed to the absence of the liver alone. These data are similar to those of Andrus, Lord and Moore (1) in respect to the rapid initial fall succeeded by a slower continuous fall. Warren and Rhoads (2) give only the terminal prothrombin values for their dogs, with the exception of two dogs hepatectomized by Mann's three stage method. In these two animals the prothrombin was 40 per cent and 45 per cent prior to the final stage. After the operation there was a definite rise for one and eight hours, respectively, followed by a progressive decrease until death.

The reaction between prothrombin A and B appears to be only partially quantitative. Our observations, in these experiments, and in others reported elsewhere, indicate that a deficiency of one component can be partially compensated for by an excess of the other. We have found previously that in plasma known to be deficient in one component but not in the other, the addi-

tion of the deficient component causes a marked decrease in the prothrombin time compared to that of a 50 per cent dilution of the plasma, while addition of the non-deficient component causes only a slight decrease. In these experiments all samples, except the last two from dog VI, showed an equivalent decrease on the addition of either prothrombin A or B. We interpret these data to mean that the decrease in prothrombin due to hepatectomy is the result of a decrease in both components and not in only one. Complete confirmation of this interpretation must await the development of precise methods of determining the concentration of each component separately. It is of interest that Quick (4) found that chloroform poisoning in dogs caused a temporary fall in both components.

In all the dogs the plasma proteins decreased markedly during the survival period. In some cases, notably dogs I and VI, this decrease was partially due to the concurrent hemorrhages previously mentioned. A comparison of values for total protein and hemoglobin, however, indicates that in none of the dogs can this decrease be ascribed solely to hemorrhage. Immediately following death large samples of blood were collected from three of the dogs by cardiac puncture. The plasmas from these samples were frozen and are being studied in the electrophoresis apparatus with the object of determining which of the electrophoretic components show marked changes. These data will be presented in a later communication. In contrast to the changes observed for the prothrombin, the fibrinogen decreased slowly during the early period following the hepatectomy and then dropped rapidly in the period shortly preceding the death of the animal.

SUMMARY

Previous observations that hepatectomy causes a decrease in the plasma prothrombin, total protein and fibrinogen have been confirmed and extended. It has been demonstrated that the fall in prothrombin is the result of a simultaneous decrease in both components of prothrombin. This observation is interpreted as evidence that the liver is concerned in the production of both of these components.

We wish to acknowledge the technical assistance of Miss Annabel Avery and Mrs. Shirley C. Northrop.

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THE EFFECT OF EXPOSURE TO OXYGEN AT HIGH PRESSURE UPON THE TONUS AND RESPIRATION OF PYLORIC MUSCLE FROM THE RABBIT¹

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Studies in this laboratory on the subject of oxygen poisoning have thus far been largely concerned with biological enzyme systems and the respiratory metabolism of isolated, surviving tissues (1). Of the series of enzymes studied, the greater number have shown a decrease in activity under oxygen at high pressure, and all of the tissues have shown some degree of decrease in oxidative metabolism, as reflected in oxygen uptake and production of CO₂. However, the most rapid effect observed has been far slower than the effect of exposure to comparable pressures upon the intact mammalian organism. We have therefore concluded that none of these phenomena represents the central mechanism of "acute" oxygen poisoning, whereby an intact rat succumbs to violent convulsions within 5 or 10 minutes of reaching an oxygen pressure of 8 atmospheres.

Bean and Bohr (2), on the other hand, using a physiological approach, reported an effect upon the function of a tissue *in vitro* whose rapidity was of the same order as that upon the intact animal. Their results and conclusions are perhaps best summarized by quotation.

Pyloric sphincter tonus is sharply decreased by exposure to oxygen at thirty and seventy-five pounds' pressure, but is unaltered by cyanide in concentrations which inactivate the oxidase-cytochrome system. Since oxygen at high pressure is known to selectively inactivate the dehydrogenase system, it is concluded that this enzyme system is more important than the oxidase-cytochrome system to that metabolism upon which the maintenance of pyloric sphincter tonus is dependent.

Oxygen at high pressure fails to cause a decrease in pyloric sphincter tonus if this muscle has been treated with NaCN prior to its exposure to the oxygen pressure. It would appear, therefore, that oxygen must be activated by a cyanide-sensitive system before it is capable of adversely affecting pyloric tonus and inhibiting the dehydrogenase system of this tissue.

In studies reported elsewhere, we have been unable, by six different types of experiments, to involve the cytochrome oxidase system in the phenomenon of the inhibition of an enzyme which occupies a central rôle in metabolic activity. For in disagreement with Libbrecht and Massart (3), we have found that succinic dehydrogenase is inactivated by high oxygen pressure in the absence as well as the presence of the cytochrome oxidase system. Furthermore, cytochrome oxidase and cytochrome C we have found to be unaffected by oxygen

¹ The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Pennsylvania.

at high pressure. In the category of dehydrogenases, we have found that xanthine oxidase is rapidly inactivated by oxygen at high pressure, succinoxidase only slowly, malic dehydrogenase negligibly, and lactic dehydrogenase not at all.

The following experiments will be seen to offer the fullest confirmation of the results of Bean and Bohr (2), but to render their explanatory hypotheses possibly incomplete, particularly in the light of the work cited above.

METHODS. The available pressure chamber, described previously (4), was not suited to the employment of the standard muscle lever or kymograph. A special apparatus was therefore built to enable tonus to be read outside the chamber, a full description of which will be given elsewhere (5). In principle, contraction of a muscle, situated within a fluid-containing tube through which gas is bubbled, is opposed by a torsion balance connected to a friction-free variable resistor whose leads run outside the chamber to one arm of a modified Wheatstone bridge. Tonus is then read on a microammeter connected across the bridge and calibrated directly in grams. In the experiments described, the sensitivity of the instrument was approximately 0.1 gram per scale division (1 mm.), with a reproducibility of ± 100 mgm. under repeated variations in pressure. The change in muscle length was 0.4 mm. per gram, the usual length of the specimens under tension being 4 cm. For the purposes of this study it was considered sufficient to record only changes in tension without introduction of the length factor since the results are considered only on a comparative basis.

Special precautions were taken to insure the presence of known, constant concentrations of cyanide in both tonus and respiration experiments. In the former, control determinations of cyanide concentration as a function of time in a tube of fluid medium through which gas was bubbled were made by the colorimetric method described by Smith (6), in which the addition of an alkaline picrate solution to cyanide results in the formation of isopurpurate. It was found, as might be expected in view of the fact that cyanide in a neutral medium exists almost entirely as volatile HCN, that there was a rapid loss. This was controlled by bubbling the gas first through a much larger volume of neutral solution containing the same cyanide concentration as the specimen tube. In the case of the respiratory measurements, the cyanide concentration was controlled by a method fully described elsewhere (7).

The procedure in the tonus measurements was as follows. A rabbit was anesthetized intravenously with Nembutal and decapitated. The pylorus was removed to saline, and mucous membrane and peritoneum were dissected away. The sphincter was cut and the muscle trimmed to approximately even cross-section. Linen thread was then passed through each of the cut ends for attachment to the apparatus. The mounted muscle was immersed in a phosphate-Ringer's medium of buffer strength 0.040 M at pH 7.2, with added glucose 0.2 per cent. Tension was adjusted to the neighborhood of 8 grams, and the apparatus mounted in the pressure chamber so that the specimen tube was two-thirds immersed in water contained in the chamber and maintained at 38°C. The chamber was closed, and oxygen was bubbled upward through the

medium in the tube by means of a 2-way stopcock at its inferior end, connected by rubber tubing to a gas inlet controlled from outside the chamber. Tonus was read on the meter at intervals small enough to record all changes—from 1 to 5 minutes—and plotted directly by means of the calibration curve of the instrument. After sufficient time had elapsed for temperature equilibration of muscle and apparatus and the attainment of even tension, the pressure was raised to 8 atmospheres in 2 minutes, maintained there for the specified time (usually 10 min.), and lowered in a length of time equal to that at high pressure. NaCN or NaN_3 were added by pipette with the chamber open at 1 atmosphere. Following exposure with cyanide or azide, the medium was drained out and replaced twice before the next exposure. Control runs with a weight hanging from the muscle and apparatus established the absence of mechanical artifacts.

Respiration was measured as follows. The pylorus was prepared exactly as above, but immediately upon dissection was cut in 0.35 mm. slices by a method previously described (8). The slices were washed in saline, lightly blotted, and weighed on a torsion balance before being transferred to the same medium as above in standard Warburg respiratory vessels. Attached to manometers, the vessels were equipped with alkali insets of 0.1 NaOH for the absorption of CO_2 . Cyanide was added directly to the medium in the Warburg vessels for determination of its inhibition of oxygen uptake without the use of preliminary control periods, since the normal rate of oxygen uptake of this tissue was found to be extremely constant. The effect of high oxygen pressure upon the rate of oxygen uptake was measured by means of the special high pressure Warburg apparatus previously described (4), following a preliminary period at 1 atmosphere, readings being plotted in the same way as at 1 atmosphere.

EXPERIMENTAL. Figure 1 shows the data of 2 representative experiments of 2 types. (In all, 8 experiments were performed.) Section (a) illustrates the last 3 of 4 successive exposures to high pressure. The original tonus at the start of the first compression was 8.15 grams, the recovery from the first exposure being incomplete (6.65 grams). Subsequent experience, however, showed almost without exception a recovery to 100 per cent of the original level, indicating that the muscle was probably not stabilized with respect to tension at the start of this experiment. The figure shows the remarkable degree of reproducibility of the tonus change associated with exposure to high oxygen pressure. It is apparent that no irreversible changes which can be detected by this method have resulted from repeated exposures which would unquestionably have resulted in deep coma and probably death in the intact animal.

In a separate experiment, the pressure was maintained at 8 atmospheres for 60 minutes. The same almost immediate decrease in tonus was observed upon compression, which was, however, not reversed in the course of 85 minutes after decompression. In this case some irreversible change had undoubtedly taken place. (Similar exposure of liver, kidney and brain slices from the albino rat, separately reported (1), resulted in an irreversible decrease in their rates of oxygen consumption.)

Section (b) of figure 1 is shown as additional evidence that the phenomenon of tonus change is beyond doubt due to the oxygen itself, and not the mechanical effects of pressure or of decompression. Exactly the same technique was employed as before with the exception of substitution of nitrogen for oxygen where indicated.

It should be noted here that the total time for the cycle of tonus change from compression to complete recovery was from 40 to 60 minutes. This fact is of possible importance when compared to the analogous time in experiments described below.

Figure 2 gives data also representative of additional experiments. The effect of high oxygen pressure is here compared in the presence respectively of NaN_3 and NaCN . The elimination by cyanide of the tonus change found in the untreated muscle is seen exactly as described by Bean and Bohr (2). Also

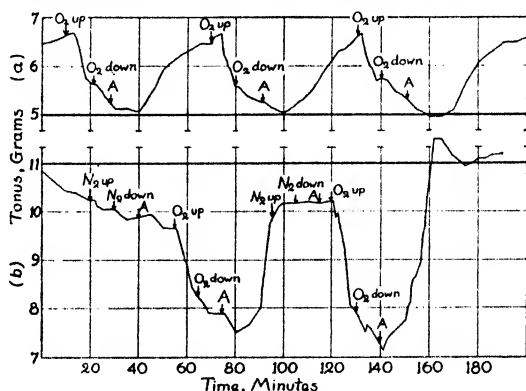


Fig. 1. Section (a): Variations of tonus of isolated pyloric sphincter muscle of the rabbit under repeated exposure to 8 atmospheres oxygen pressure. The time required for compression was 2 minutes. Decompression was begun 10 minutes after the start of compression. Atmospheric pressure was reached at points marked A. Section (b): Variations in tonus under alternate exposures to 8 atmospheres of nitrogen and oxygen. The same conditions and symbols apply as in section (a).

in confirmation of Bean and Bohr (2), with higher concentrations of cyanide, there was a distinct increase in tonus, in one case amounting to 30 per cent. In the presence of azide, however, whose effect upon enzyme systems is discussed below, the result appears to differ from that in the untreated muscle, as well as that in the presence of cyanide. While the degree of change in tonus appears greater than in the case of the untreated muscle, it is doubtful whether this is significant in view of the variation in sensitivity between preparations. The total time of the cycle, however (conditions of compression and decompression having been identical with those of the first experiment), is in this case reduced to 25 minutes. The significance of this finding is not at once apparent. In any case, it is evident that there was no prevention of the change in tonus such as that produced by cyanide.

Figure 3 summarizes the data on the oxygen uptake of pyloric muscle slices.

The upper curve shows that a 30 minute exposure to 8 atmospheres oxygen pressure produces a small, gradual decrease in oxygen uptake, which can only be considered significant at the end of this time. This result is much the same as that obtained under similar conditions with rat diaphragm (1). On the other hand the presence of cyanide at $\frac{1}{30}$ the concentration used in the tonus experiments (0.001 M) inhibited the oxygen uptake to 30 per cent of the mean control rate.

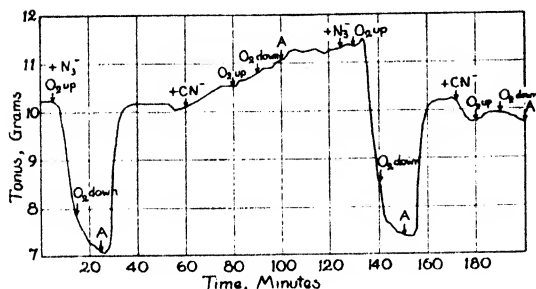


Fig. 2

Fig. 2. Variations in pyloric sphincter tonus under repeated exposure to 8 atmospheres oxygen pressure in the presence of NaN_3 0.03 M or NaCN 0.03 M. NaN_3 or NaCN were introduced into the medium 10 minutes before compression, and washed out after return to the original tonus level by 2 changes of medium.

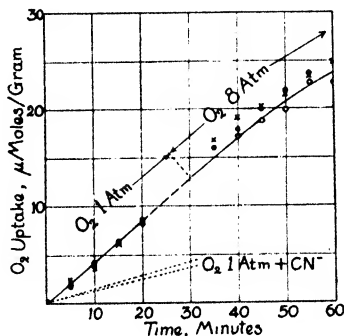


Fig. 3

Fig. 3. Oxygen consumption of 0.35 mm. slices of pyloric sphincter muscle in glucose-phosphate-Ringer's medium, expressed as micromoles per gram wet weight. Upper curve: Oxygen uptake of 3 preparations at 1 and then 8 atmospheres oxygen pressure. Lower curves (broken lines): oxygen uptake of 2 preparations in the presence of NaCN 0.001 M, at 1 atmosphere oxygen pressure.

DISCUSSION. The failure to regain tonus resulting from prolonged exposure to high oxygen pressure probably represents a true toxic effect of oxygen but should be distinguished from the acute, or immediate effects. That high oxygen pressure produces an immediate change in the tonus of this particular muscle *in vitro* there can be no doubt, but the fact that 3 or more successive exposures of the same preparation in a period of 3 or 4 hours causes no permanent change leaves the assignment of the phenomenon to the category of *oxygen poisoning* still open to question.

In agreement with Bean and Bohr (2), one must conclude from these experiments with cyanide, as well as theirs, that tonus is in this case maintained by a cyanide-insensitive system. It also appears that there is present a cyanide-sensitive mechanism, without which the effects of high oxygen pressure are not apparent. Furthermore, the inhibition of oxygen uptake of the muscle by cyanide and the absence of its inhibition by high oxygen pressure within 30 minutes must be taken to indicate that tonus is maintained at least facultatively by metabolic processes not reflected in the oxygen uptake, as measured by standard methods.

In general, it is clear that the results of these experiments are in perfect accord with those of similar, and in some cases, identical experiments by Bean and Bohr (2). However, the explanatory hypotheses offered by them are opened to question by three sets of observations.

In the first place, Keilin has demonstrated conclusively that azide inhibits cytochrome oxidase as well as other systems to almost the same degree as equivalent concentrations of cyanide. The mechanism of the inactivation of cytochrome oxidase by azide has been shown by Keilin (9) and later Winsler (10) to differ from that of its inactivation by cyanide, and the degree of inactivation has been found to be lower. However, the experiments reported have shown that azide produces if anything, an increase in the rate and extent of tonus loss under high oxygen pressure. This result appears to be incompatible with the hypothesis that tonus loss is in this case dependent upon the "activation" of oxygen by the cytochrome system. The prevention of tonus loss by cyanide must then be assumed to be due to some mechanism not involving the cytochrome system, but otherwise undefined.

Secondly, the other work in this laboratory cited above has failed to implicate the cytochrome oxidase system in the inactivation of an important enzyme, viz., succinic dehydrogenase by high oxygen pressure.

Finally, Bean and Bohr (2) have said, "The tonus fall induced by the high oxygen pressure is, perhaps, best explained by what amounts to a 'hyperoxic anoxia'—a failure of utilization of oxygen due to the poisoning or inactivation of the dehydrogenase system by oxygen in high concentrations." While perfectly possible, this hypothesis seems unlikely in view of the fact that there was essentially no change in the oxygen uptake of pyloric sphincter muscle under high oxygen pressure, indicating essentially no failure in its utilization of oxygen.

In general, the experiments here reported, together with others from this laboratory, confirm the observations of Bean and Bohr (2) but lead to so many difficulties of interpretation that the definitive hypotheses offered by them appear premature.

SUMMARY

1. In confirmation of Bean and Bohr, the tonus of the pyloric sphincter muscle of rabbits is found to be depressed by exposure to oxygen under high pressure at a rate similar to that at which the intact animal succumbs under similar conditions to convulsions and death.

2. This effect is not reproduced under nitrogen at high pressure.

3. The loss of tonus is completely prevented when the muscle is exposed at high pressure in the presence of NaCN in concentrations sufficient to inhibit respiration of the muscle 70 per cent.

4. The loss of tonus is not prevented by the presence of NaN_3 in concentrations considered sufficient to inactivate the cytochrome oxidase system to the same degree as cyanide.

5. The total oxygen uptake of the muscle is not significantly affected by high oxygen pressure during the time at which its tonus is depressed.

6. The implication of the cytochrome oxidase system in the phenomenon, and the possible rôle of some form of anoxia are shown to be open to question.

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EFFECT OF AMBIENT AIR TEMPERATURE AND OF HAND TEMPERATURE ON BLOOD FLOW IN HANDS¹

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There have been several recent descriptions of the effects of the general thermal condition of the body and of the temperature of hands on blood flow through hands (Abramson, 1944; Burton, 1939; Freeman, 1935). However, there is still lacking a comprehensive quantitative description of the volume flow of blood in hands for the range of conditions that may exist in everyday life.

In a previous report, the author (1945) summarized preliminary results of blood flow determination in hands maintained at temperatures varying from 10 to 35°C. These studies, made under conditions such that the body as a whole was comfortable, have been extended, and similar data have been obtained in uncomfortably warm and uncomfortably cold environments.

EXPERIMENTAL CONDITIONS AND PROCEDURES. Blood flow measurements were obtained on lightly dressed subjects (men, 18 to 35 years of age) sitting at rest in a room maintained at temperatures such that they were uncomfortably warm (32°C., dry bulb; 28°C., wet bulb), comfortable (24°C., dry bulb; 19°C., wet bulb) or uncomfortably cold but not shivering (16°C., dry bulb; 13°C., wet bulb). The subjects kept one hand in a plethysmograph through which water at any desired temperature between 2 and 35°C. was circulated. Experiments lasted three hours. During this time, determinations of the volume of blood circulating through the hand were made and surface temperatures of the hands, feet and trunk were recorded.

Blood flow determinations were made using the venous occlusion technique of Hewlett and Van Zwaluwenburg (1909). The apparatus was conventional in design except that the plethysmograph was made of glass, which allowed the hand to be observed, and that the connection between the plethysmograph and the recording spirometer consisted of a glass manometer, permitting accurate adjustment of pressure. Accuracy of measurements was estimated by introducing water at known rates into the plethysmograph with a subject's hand in place and in comparing these values with values actually recorded. In 84 such tests, 63 of the determined values were within 25 per cent of the correct values. However, there was a tendency for determined values to be somewhat high when flow was small and slightly low when flow was great. Correction of blood flow determinations for these trends was not made, since more serious errors probably occur during actual blood flow measurements.

Surface temperatures of the chest, back, great toe, and index finger of each

¹ The material in this article should be construed only as the personal opinion of the writer and not as representing the opinions of the Navy Department officially.

hand were obtained with thermocouples attached with adhesive tape. The surface temperatures obtained on the index fingers of hands immersed in water were probably not very exact in experiments with colder water in which a large temperature difference between fingers and water existed. If the fingers and water participated equally in determining the temperatures recorded by the thermocouples, true surface temperature of fingers in water at 2°C., for example, would be 12.6°C. instead of the recorded value, 7.3°C. (see table 2). However, the thermocouples were no doubt influenced more by fingers than by water, since they were bound closely to the fingers with adhesive tape. Therefore, it

TABLE 1

Average blood flow (cc./100 cc. hand volume/min.) in hands immersed in water at the temperatures indicated, obtained under environmental conditions such that subjects were uncomfortably warm (32°C., D.B.; 28°C., W.B.), comfortable (24°C., D.B., 19°C., W.B.) and uncomfortably cold (16°C., D.B., 13°C., W.B.). Extreme values are given in parentheses. These values are averages of twelve determinations made during the third hour in experiments lasting three hours. Temperatures obtained on the index finger of hands immersed in water are also given, although they are known to be rather inexact in the experiments in colder water (see text). All temperatures are in °C.

T (WATER)	2	5	10	15	20	25	35
A. Body uncomfortably warm (3 subjects)							
T (finger).....	7.3	11.3		16.7	22.2		35.2
Blood flow.....	6.4	6.8		5.5	8.1		20.6
	(4.5-8.2)	(4.9-10.4)		(3.8-8.7)	(5.9-9.6)		(17.3-25.4)
B. Body comfortable (6 subjects)							
T (finger).....		8.3	11.9	15.5	20.5	25.5	35.1
Blood flow.....		4.3	2.5	0.9	1.3	2.7	5.9
		(3.1-8.0)	(0.8-4.3)	(0.3-1.5)	(1.0-1.7)	(1.4-4.4)	(2.7-9.6)
C. Body uncomfortably cold (3 subjects)							
T (finger).....			11.1	15.0		25.1	35.0
Blood flow.....			1.9	0.3		0.6	1.9
			(1.0-3.7)	(0.2-0.5)		(0.3-0.7)	(1.5-2.5)

seems likely that the surface temperature of the fingers in this case was between 7.3 and 12.6°C. This is the most extreme case. Finger temperatures in other experiments are known more accurately.

RESULTS. In the comfortable environment, blood flow was less in moderately cold hands (0.9 cc./100 cc. hand volume/min. for hands in water at 15°C.) than in very cold hands (4.3 cc./100 cc./min. for hands in water at 5°C.) or in warm hands (5.9 cc./100 cc./min. for hands in water at 35°C.). A qualitatively similar relationship existed in the uncomfortably cold environment. In the uncomfortably warm environment, however, blood flow in moderately cold hands (water temperature, 15°C.) was about the same as in very cold hands

(water temperature, 2°C.). At any given hand temperature, blood flow was greater the warmer the body. The effect of body warmth was most pronounced in moderately cold hands (ca. 15°C.) where blood flow in the case of the uncomfortably warm environment was about twenty times that in the uncomfortably cold environment, and least pronounced in very cold hands (ca. 10°C.) where the difference was only about threefold (table 1).

These blood flow values are averages of twelve determinations made at five-minute intervals during the third hour when equilibrium conditions were fairly

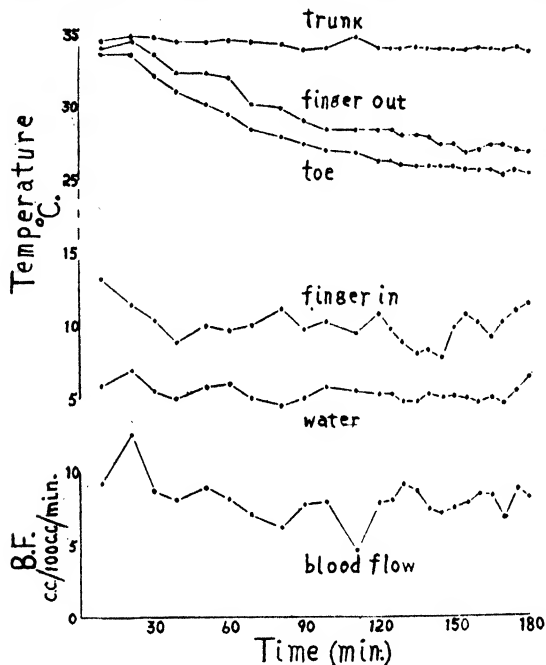


Fig. 1. Typical data obtained on one subject at a room temperature of 24°C., dry bulb; 19°C., wet bulb. Surface temperatures of the trunk and extremities show that approximately two hours are required for establishment of thermal equilibrium between the body and environment (upper three curves). The progressive decrease in blood flow during the first hour or so occurred in most instances (lowest curve). The temperature of the water used in the plethysmograph and of the hand on which blood flow measurements were made (finger in) are also recorded (middle two curves).

well established, as judged by blood flow and skin temperature measurements. Blood flow decreased during the first hour or so in most experiments (fig. 1).

The general thermal condition of the subjects was reasonably constant during the third hour in all experiments at any one environmental temperature. However, the temperature of the water in the plethysmograph exerted some influence since surface temperatures of the trunk and extremities tended to be slightly higher the warmer the water (table 2). The relatively low trunk temperature (34.6°C.) in the uncomfortably warm environment when hands were in

water at 35°C. probably was caused by greater evaporative cooling resulting from increased sweating; it may be noted that finger and toe temperatures were high (36°C.).

DISCUSSION. There do not appear to be many published data with which our results can be compared. Freeman (1935) has made one of the most extensive studies of the relationship between the temperature of hands and blood flow through hands, but he used only one subject in obtaining the data showing the relationship. Many blood flow measurements have been made in comfortable environments with hands maintained at a temperature of 30 to 35°C. Representative results are those of Abramson and Fierst (1942) who found an

TABLE 2

Mean values of temperatures (°C.) obtained on the trunk, fingers and toes of subjects sitting in uncomfortably warm, comfortable, and uncomfortably cold environments while holding one hand in a plethysmograph filled with water at the temperatures indicated. These measurements were made during the third hour of experiments.

BODY AREA	WATER TEMPERATURE							Mean
	2	5	10	15	20	25	35	
A. Body uncomfortably warm; room temp. 32°C., D.B.; 28°C., W.B.								
Trunk.....	35.6	35.6	35.7	35.5	35.7		34.6	35.5
Finger.....	35.0	35.1	35.6	35.8	36.0		36.0	35.6
Toe.....	34.6	34.8	35.3	36.2	36.1		36.0	35.5
B. Body comfortable; room temp., 24°C., D.B.; 19°C., W.B.								
Trunk.....		34.6	34.5	34.7	34.8	34.3	34.6	34.6
Finger.....		28.5	32.3	31.3	33.8	33.3	33.0	32.1
Toe.....		28.2	31.0	31.6	32.8	32.5	32.4	31.3
C. Body uncomfortably cold; room temp., 16°C., D.B.; 13°C., W.B.								
Trunk.....			32.3	33.4		32.9	31.4	32.5
Finger.....			19.3	21.4		20.9	20.9	20.6
Toe.....			18.7	19.5		18.7	19.1	19.0

average value of 9.3 cc./100 cc./min. in a study of 61 subjects. Measurements made by Freeman et al. (1936) in a series of 15 subjects indicate an average value of 6.8 cc./100 cc./min. An average value of 5.9 cc./100 cc./min. was obtained under similar conditions in the present study. There do not appear to be any data on blood flow in extremely cold hands, except for some preliminary observations reported by us (1945). Lewis (1929-31) had previously inferred that blood flow was greater in very cold extremities than in moderately cold ones, but he did not make any blood flow measurements. Studies of the influence of the general thermal condition of the body on blood flow have been made occasionally (e.g., Abramson et al., 1939; Freeman et al., 1936); however, experimental conditions are not usually defined clearly enough to permit comparison with results of our study.

SUMMARY

1. Measurements of blood flow in hands were made on young men (18 to 35 years of age) sitting in uncomfortably warm, comfortable, and uncomfortably cold environments with hands immersed in water at temperatures ranging from 2 to 35°C.

2. Under comfortable environmental conditions, blood flow was less in moderately cold hands (0.9 cc./100 cc./min. for hands in water at 15°C.) than in very cold hands (4.3 cc./100 cc./min. for hands in water at 5°C.) or in warm hands (5.9 cc./100 cc./min. for hands in water at 35°C.). A qualitatively similar relationship existed in uncomfortably warm and uncomfortably cold environments. In the uncomfortably warm environment, however, blood flow in moderately cold hands (water temperature, 15°C.) was about the same as in very cold hands (water temperature, 2°C.)

3. At any given hand temperature, blood flow was greater the warmer the body. This effect was most pronounced in moderately cold hands (ca. 15°C.) where blood flow in the case of the uncomfortably warm environment was about twenty times that in the uncomfortably cold environment, and least pronounced in very cold hands (ca. 10°C.) where the difference was only about three fold.

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THE EFFECTS OF INTRACISTERNAL INJECTION OF POTASSIUM PHOSPHATE ON THE RATE AND RHYTHM OF THE HEART AND ON THE BLOOD PRESSURE AND ON THE RESPIRATION OF THE DOG

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Several studies have been made on the effects of increasing the K:Ca ratio in the cerebrospinal fluid and in the medullary circulation. A marked increase of the blood pressure and an increase of the heart rate have been reported and these observations have been confirmed consistently. On the other hand, slowed heart rate and decreased blood pressure usually are not reported. Furthermore, some observers have reported inhibition of respiration; others, stimulation; still others, both inhibition and stimulation.

Stern and Chvoles (1933) observed a marked increase of the blood pressure and an augmentation of the heart rate and the respiration after intraventricular injection of KCl in the dog. Similar results were obtained with Na_2HPO_4 . After observing the effects of intracisternal injection of potassium phosphate in the dog Stern (1942) concluded that by direct action the ionic K stimulates the sympathetic nerve centers and that the phosphate, by reducing the concentration of ionized Ca, causes a depression of the parasympathetic center tone. Smolik (1944) reported marked rises in blood pressure and acceleration of pulse rate after intracisternal injection of potassium phosphate in dogs. The respiratory rate increased after an initial inhibition. Results of intracisternal injection of potassium phosphate in the rabbit similar to those observed by Smolik were reported by Downman and Mackenzie (1943). They also observed a slowing of the pulse and a fall of the blood pressure which were not altered by vagotomy. Resnik, Mason, Terry, Pilcher and Harrison (1936) found that intracisternal injection of KCl in small amounts decreased the blood pressure in dogs. Larger amounts caused an increase in blood pressure. Respiration was usually increased. In 1938 von Euler reported that the injection of KCl into the internal carotid artery or vertebral artery of the cat decreased the heart rate and blood pressure and caused a transient inhibition of respiration. Intracisternal injection of KCl produced a marked rise in blood pressure and very little change in respiration. Hooker (1915) perfused the medulla of the dog and reported that solutions with a high K:Ca ratio inhibited the respiratory center and slowed the heart rate.

It is obvious that the reports upon the effects of increasing the K:Ca ratio in the medullary centers are not in agreement. Furthermore, the studies using this method of stimulation have failed to report observations on the cardiac activity and they did not discriminate the changes in the expiratory and inspiratory phases of respiration.

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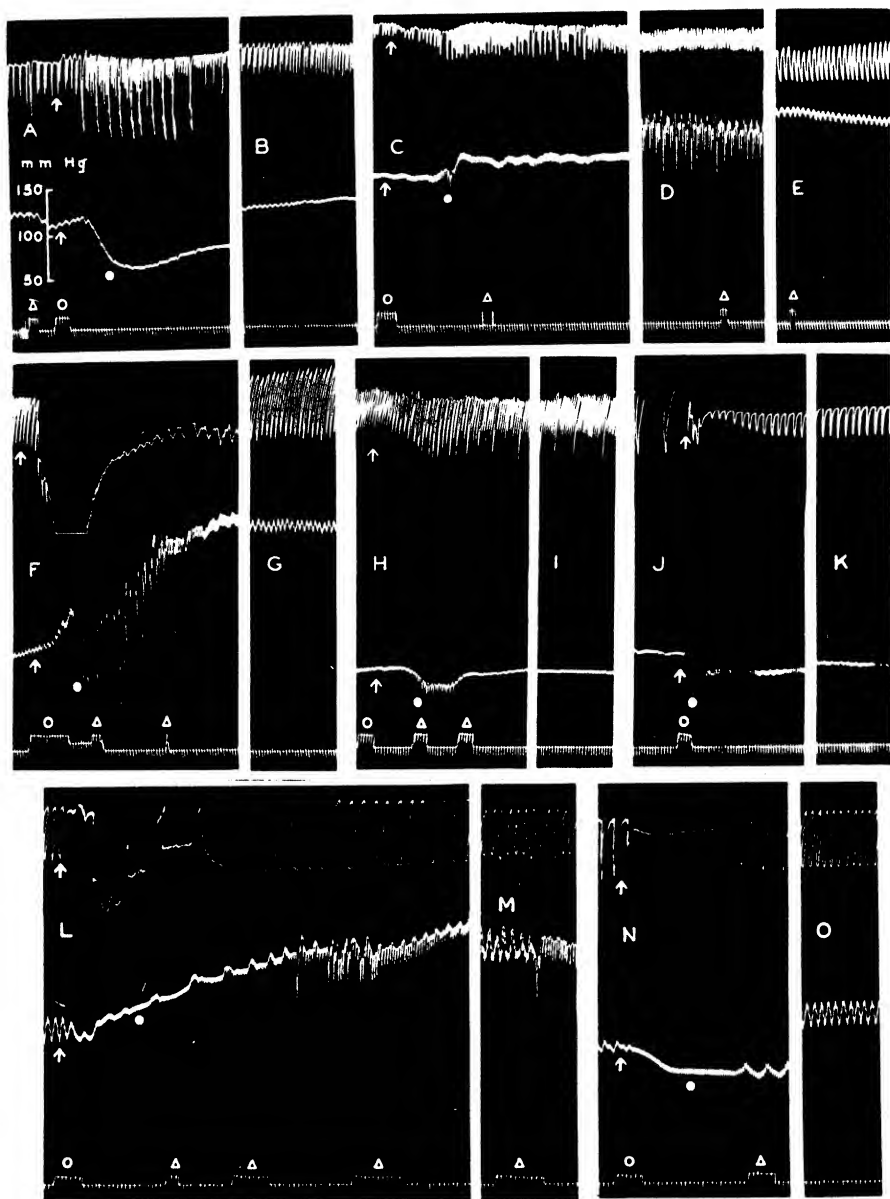


Fig. 1. The effect of intracisternal injection of potassium phosphate on respiration (uppermost tracing) and blood pressure (second tracing). The lowest tracing shows time in seconds and zero blood pressure. The calibration shown in A is applicable to all blood pressure records in the figure. The beginning of injection (arrows), the period of injection (circles), the period of electrocardiographic recording (triangles) and the moment of greatest initial slowing of heart rate (white dots), are indicated. Inspiration is the down-stroke.

A. Intact dog, first injection of 0.03 cc./kgm. Heart rate decreased from 180 to 145. B. Heart rate 160 $1\frac{1}{2}$ min. after A. C. Intact dog, second injection of 0.03. Heart rate

The purpose of this study is to investigate the effects on heart rate, blood pressure and respiratory rate of the intracisternal injection of potassium phosphate and in addition to make observations on the changes in the cardiac rhythm and on the changes in the expiratory and inspiratory responses of the respiration.

METHOD. One-sixth molar mono- and di-potassium phosphate were mixed and brought to a pH of 7.6 using a pH meter of the Leeds and Northrup universal-indicator type. The solution was injected into the *cisterna magna* after removal of a volume of cerebrospinal fluid equal to the volume to be injected. Usually a period of 15 to 20 minutes was allowed to elapse between successive injections. Mongrel dogs were used in these experiments which were carried out under barbital anesthesia given intravenously (260 to 270 mgm. per kgm. of body weight). In the group of animals given intracisternal injection of potassium phosphate 4 were left intact, 4 were vagotomized before injection, 4 were vagotomized after various amounts had been injected, the spinal cord was sectioned at the level of the sixth cervical vertebra in 3, transection of the brain stem was made in the intercollicular region in 2 and in the anterior pontile region in 1. Potassium phosphate solution was applied directly to the floor of the fourth ventricle in 1 dog. One-sixth molar CaCl_2 was injected into the *cisterna magna* of 2 dogs. The brain stem was exposed for decerebration by removal of the occipital and parietal lobes on one side. Using the tentorium as a guide, the brain stem was sectioned with a sharp cutting instrument. The brains were then fixed for two weeks in formalin and the level of section verified by gross examination.

A differential amplifier was used to obtain the electrocardiograms. The ground lead was placed above the sternum and the active leads were inserted near the axilla on the right side and near the lower boundary of the chest on the left side. Respiratory movements were recorded from a thoracic cage pneumograph and Marey's tambour. Two spirometer recordings were made.

For convenience of presentation the more frequently used amounts of potassium phosphate solution, 0.03 cc. and 0.12 cc. per kgm. of body weight, will be called, respectively, a small dose and a large dose. The dosage, when expressed numerically, will designate the number of cubic centimeters of M/6 potassium phosphate per kilogram of body weight.

RESULTS. 1. *Blood pressure and heart rate.* When small doses of potassium phosphate were given to the intact animal the blood pressure fell and then returned to normal or slightly above normal (fig. 1A and B). Subsequent small

decreased from 136 to 85. D. Tracing 8 min. after C; heart rate 88. E. Record 1 min. after D showing the effect of vagotomy; heart rate 102. F. Intact dog given 2 doses of 0.03 followed by 1 of 0.12. The initial slowing of the heart rate from 160 to 40 was followed by an increase to the pre-injection rate. G. Heart rate 240 three minutes after F. H. Dog with spinal cord section at C_6 , first injection of 0.03. Heart rate decreased from 116 to 68. I. After 1 min. in same animal; heart rate 140. J. Dog with spinal cord section at C_6 , second injection of 0.03. Heart rate decreased from 112 to 40. K. After 2 min. in same animal; heart rate 116. L. Vagotomized dog, injection of 0.12 subsequent to 0.03. Heart rate decreased from 205 to 178. M. Record 6 min. after L; heart rate 248. N. Vagotomized dog, first injection of 0.03. Heart rate decreased from 185 to 140. O. Record 3 min. after N.; heart rate 202.

doses produced no decrease of the blood pressure (fig. 1C). After large doses were injected it increased sharply and remained at a high level for 10 to 15 minutes (fig. 1F and G). The blood pressure remained high after repeated injections and each successive injection produced a definite but progressively less marked rise. The blood pressure fell little or not at all when small doses were given to vagotomized animals and 4 minutes after injection it had risen 30 per cent or more above the pre-injection level. Large doses were followed by a rapid increase of the blood pressure to a level somewhat higher than that obtained in intact animals (fig. 1L to O and fig. 2A to D). Injection of small doses of potassium phosphate in dogs with section of the spinal cord at C₆ produced an initial transient fall of the blood pressure which was followed by a slow rise to

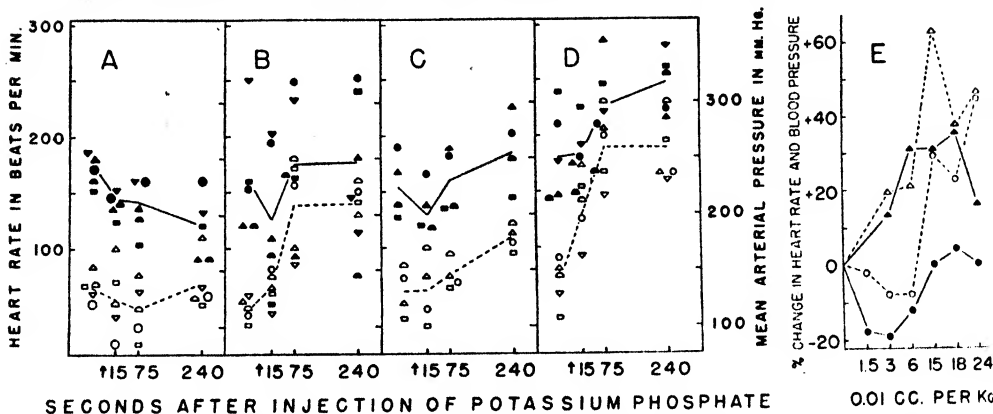


Fig. 2. A comparison in vagotomized and intact dogs of the effects of intracisternal injection of potassium phosphate on heart rate and blood pressure. Solid lines: average heart rate; broken lines: average blood pressure.

A to D. Solid symbols: heart rates; corresponding open symbols: corresponding blood pressures. A. Intact, 0.03 cc./kgm. B. Intact, 0.12. C. Vagotomized, 0.03. D. Vagotomized, 0.12. E. In this chart is shown the per cent of increase or decrease in the heart rate and blood pressure 15 min. after each injection. The zero of the ordinates is established from the pre-injection levels. The abscissae designate the total amount of potassium phosphate injected prior to each reading. Triangles: averages in vagotomized dogs; circles: averages in intact dogs.

the pre-injection level (fig. 1H and I). No significant increase of the blood pressure was observed in these animals after large doses.

The injection of small doses produced an initial decrease in the heart rate (fig. 1, white dots) which remained slow in the intact animals, but increased to a rate which exceeded the pre-injection level in the animals in which the vagi had been cut and in those in which the spinal cord had been sectioned. The heart rate usually fell below and then rose above the pre-injection rate when large doses were given to intact animals. In some animals the rate remained high, in others marked bradycardia appeared less than 4 minutes after the injection. Usually there was little or no decrease in heart rate following large doses in vagotomized dogs. The rate increased markedly and remained high for 15 to

20 minutes after the injection. In figure 2A to D are shown typical effects on blood pressure and heart rate when small and large doses are given to intact and vagotomized animals.

The heart rate and blood pressure tended to level off and undergo little change 10 to 15 minutes after injection. Successive injections increased the heart rate and blood pressure more rapidly in the vagotomized dogs than in those left intact. As the dosage approached the level at which respiration showed signs of failure the heart rate and blood pressure in the latter more closely approximated the level obtained in the former group of animals. The arithmetical means for blood pressure and for heart rate 15 minutes after each injection are shown in figure 2E for all dosages used in vagotomized and intact dogs.

The intracisternal injection of potassium phosphate after transection of the brain stem in the intercollicular region and in the anterior pontile region produced changes in heart rate and blood pressure which were similar to the effect obtained in intact dogs receiving comparable doses.

2. *Cardiac activity.* Because the intracisternal injection of potassium phosphate so often resulted in irregularities of the blood pressure record, apparently due to cardiac arrhythmias, electrocardiograms were obtained. The records show a variety of abnormalities, including scattered ectopic beats, cycles of alternating form (bigeminal rhythms), nodal and ventricular rhythms. These arrhythmias usually appeared 1 to 2 minutes after the injection of the solution in intact and vagotomized animals. Section of the spinal cord at the level of the sixth cervical vertebra ended the appearance of such abnormalities in response to injection of the solution though they could still be elicited by tetanic stimulation of the spinal cord just below the level of section or by stimulation of one or the other stellate ganglia, especially the left. Brain stem transection did not prevent the induction of arrhythmias, ectopic beats and abnormal forms of complexes by intracisternal injection of potassium phosphate. Figure 3 indicates the variety of electrocardiographic changes obtained in the course of experiments on intact and vagotomized dogs. Records were traced for purposes of reproduction. Changes in cardiac rhythm and forms of complexes were frequently related to the inspiratory and expiratory phases of respiration. However, after marked tachycardia appeared (fig. 3C) no cardiac changes associated with the respiratory cycle were observed. The sinus arrhythmia shown in figure 3A is typical of the effect produced when small doses were given to intact dogs. Small doses rarely elicited sinus arrhythmia in vagotomized animals though ectopic beats frequently occurred during inspiration (fig. 3D and D'). In figure 3B is shown the superimposed effect of respiration upon a bigeminal rhythm established about 2 minutes after the injection of a large dose. The intervals between normal cardiac cycles decrease during inspiration. The sinus arrhythmias appearing after large doses in vagotomized dogs were less marked than those produced by similar doses in intact animals. Moreover, a greater variety of arrhythmias and changes in form of complexes were seen in the vagotomized dogs (fig. 3E to H). The complexes of abnormal form and ectopic beats were related to the inspiratory phase of the respiratory cycle. Normal cardiac cycles appeared more frequently during expiration.

3. *Respiration.* The responses of the respiration to intracisternal injection of potassium phosphate may be separated into two groups on the basis of the

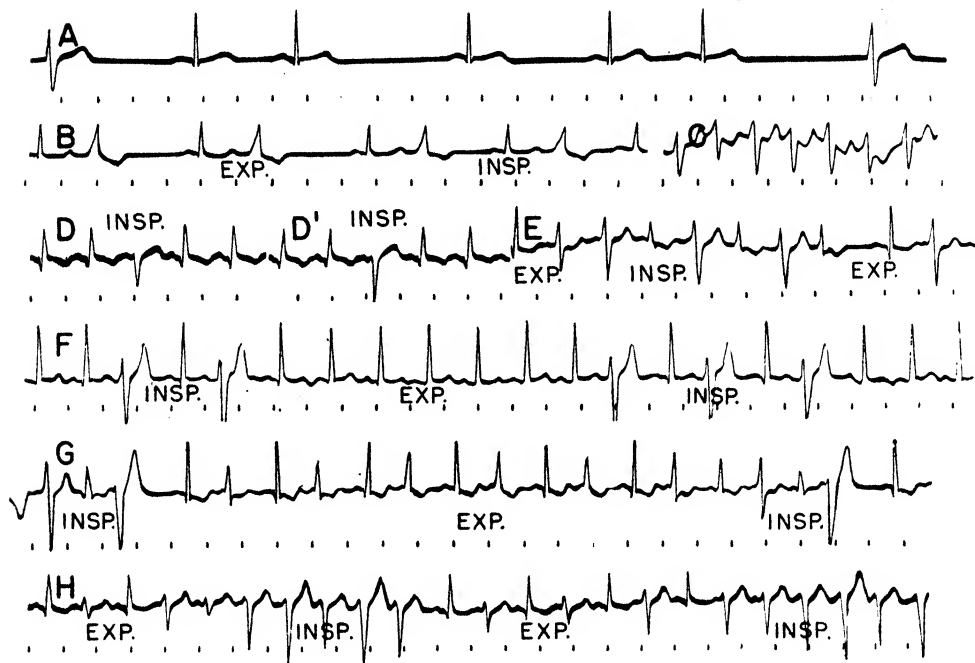


Fig. 3. Electrocardiograms from intact and vagotomized dogs illustrating changes which followed intracisternal injection of potassium phosphate. Time intervals 0.2 sec. The original records were traced for reproduction.

A. Intact animal given a small dose. The record, which covers a period of somewhat over 4 respiratory cycles, shows marked sinus arrhythmia. In the first and last cycles, ectopic beats are related to respiratory movements with deep inspiratory phase. B. Intact animal given 2 small doses and 1 large dose. There is a bigeminal rhythm and respiratory arrhythmia, the intervals between normal beats decreasing during inspiration though the intervals between a normal cycle and the subsequent abnormal cycle remain almost constant. C. Intact animal, 2 large doses. Tachycardia with irregularities. D and D'. Vagotomized animal 1 small dose. Record of nine similar cardiac cycles removed between D and D'. Heart rate rapid and respiratory rate slow. During inspiration a frankly ectopic beat is released. E. Vagotomized animal given 3 small doses and 1 large dose. During inspiration there is a bigeminal rhythm with all complexes of abnormal form. During expiration there are 1 or 2 normal cycles. F. Vagotomized animal. One small dose and 1 large dose. Two or 3 ectopic beats alternate with normal cycles during inspiration. G. Vagotomized animal given 3 doses of 0.015 and 1 of 0.12. Bigeminal rhythms both during inspiration and during expiration but rate slightly faster and point of initiation of beat shifted during inspiration.

latent period and the type of response. The first response appeared about 1 second after the beginning of the injection and consisted of active expiration (figs. 1A, 4A and 4D), reduced amplitude of respiration (figs. 1C and 4E), or apnea in

the passive expiratory phase of respiration (figs. 4B and 4C). If inspiratory movement had begun during the interval of 0.5 to 1 second after the beginning of an injection, inspiration was interrupted by an expiratory movement (fig. 4C). The second response was seen about 5 to 6 seconds after the beginning of the injection and it could be identified by an increase of inspiratory reaction. In animals receiving small doses the amplitude of respiration began to increase gradually (fig. 1A and C). Within a brief period the depth of respiration exceeded the pre-injection level. In experiments in which the initial response

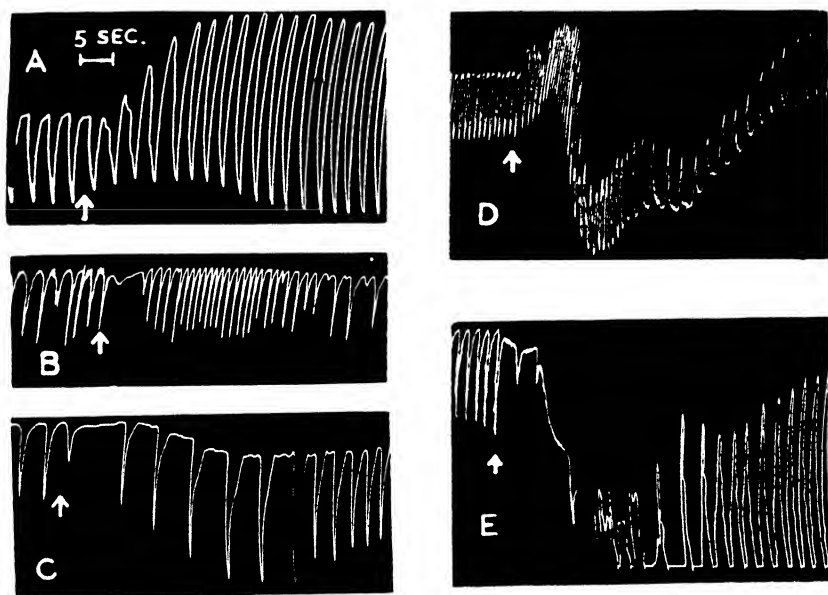


Fig. 4. Pneumographic tracings of respiratory response to intracisternal injection of potassium phosphate in the intact dog. The beginning of injection is indicated by arrows. Inspiration is the downstroke. A and B. First injection of 0.03 cc./kgm. C. Second injection of 0.03. D. Injection of 0.06 subsequent to 2 injections of 0.03. E. First injection of 0.12.

was apnea, respiration was usually resumed after a period of about 6 seconds and the typical increase in amplitude followed (fig. 4B and C).

When large doses were given the initial respiratory response was similar to the effect produced by small doses. The first effects of large doses were followed, after an interval of about 5 seconds, by strong inspiratory movements frequently sustained for several seconds (fig. 1L).

In figure 4 are shown the differences of respiratory response to the administration of doses varying from 0.03 to 0.12 cc. per kgm. The amplitude of respiration was always diminished in the first respiratory cycle which followed an injection. Frequently the midposition of the subsequent respiratory cycles was displaced toward the expiratory side (fig. 4A). Following a latent period of about 6

seconds the midposition of the respiration began to return toward the inspiratory side and the depth of respiration increased. In figure 4B another reaction to a small dose is shown. The first inspiration after the beginning of injection was interrupted and a 5-second period of apnea occurred before respiration was resumed. The first respiratory cycle was shallow being followed by cycles that gradually increased in depth. The effects of gradual increase of dosage on the depth of inspiration are shown in records 4C, D and E. An increase in amplitude of respiration and a displacement of the midposition toward the inspiratory side were produced by the second small dose (fig. 4C). After an injection of 0.06 cc. the midposition of respiration was displaced first toward the expiratory side and then toward the inspiratory side. Sustained inspiration of short duration occurred during the return of the midposition to the pre-injection level (fig. 4D). The typical results of the injection of a large dose are shown in figure 4E. Marked inspiration followed a 5-second period in which respiratory depth was diminished. Inspiration was sustained for a brief period and after respiratory cycles reappeared the inspiration was prolonged during a few subsequent cycles. A more pronounced inspiratory response is shown in figure 1F. In no experiment with the intact dog was the inspiratory phase of the cycle prolonged to the extent observed in vagotomized animals (fig. 1L).

The early effects of the injection of potassium phosphate described above were followed by increased respiratory activity which continued for many minutes. The average increase in rate 15 minutes after the injection of the first small dose was 67 per cent in intact dogs and 45 per cent in those with the vagi sectioned. The amplitude of respiration increased about 100 per cent in intact and vagotomized dogs during the first minute after injection and gradually diminished approximately to the pre-injection level by the end of a 15-minute period. Typical changes in rate and depth are shown in figure 1 at 1 to 8 minutes after injection.

The early responses of respiration to potassium phosphate in animals with the spinal cord sectioned were not as clearly defined as those in intact dogs. However, changes in rate and depth of respiration over a 15-minute period were similar to the changes seen in intact animals.

Apneustic respiration similar to that described by Pitts, Magoun and Ranson (1939b) occurred about 30 to 60 seconds after the beginning of injection. Sustained inspiration of long duration (figs. 1L and 5B) was observed frequently in vagotomized animals when either small or large doses were administered. This type of respiration was seldom produced in intact dogs and the duration of sustained inspiration was brief (fig. 4D). In figure 5A, B and C are shown the maximal apneuses which followed each of 3 successive injections in a vagotomized dog. The second small dose was followed by prolonged inspiratory movements (fig. 5B). The prolongation of inspiration was not so marked after injection of the subsequent large dose but the amplitude of respiration showed a greater increase and active expiration appeared (fig. 5C). In contrast the apneusis which followed repeated large doses was sustained at a diminished amplitude and no indication of active expiration was in evidence (fig. 5D and E). In figure 5F is shown the type of respiration following the administration of a large

injection in a dog with intercollicular transection of the brain stem. After bilateral vagotomy in this animal the duration of inspiration increased more than

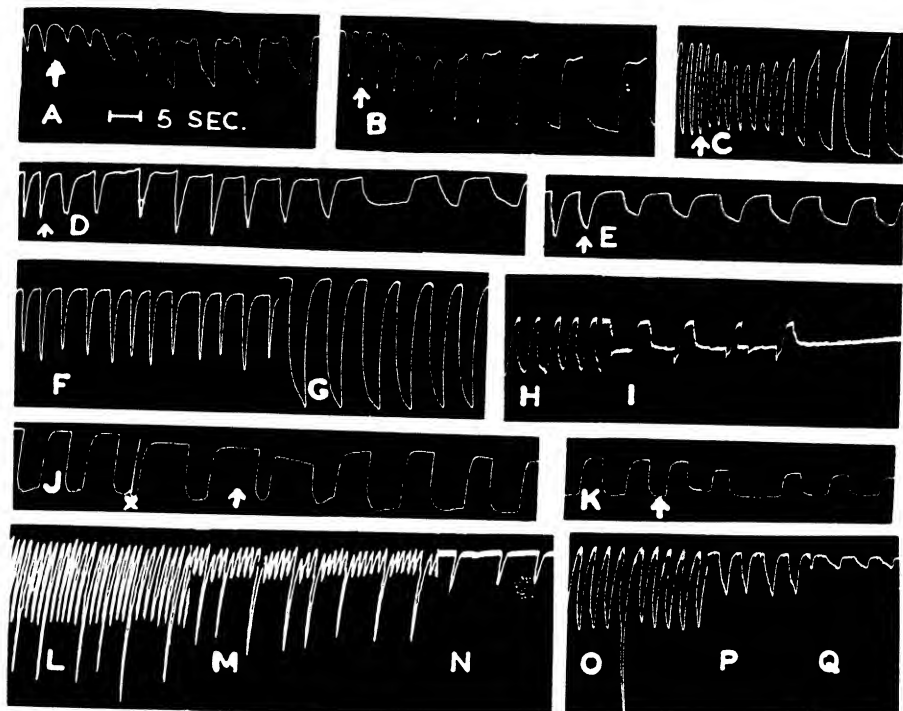


Fig. 5. Apneustic respiration and respiratory failure following the intracisternal injection of potassium phosphate. Arrows indicate the beginning of injection. Inspiration is the downstroke. A, B and C. Records of apneusis showing the greatest extent of sustained inspiration after successive injections in a vagotomized dog. A. After the first injection of 0.03 cc. kgm. B. After the second injection of 0.03. C. After the subsequent injection of 0.12. D. Apneusis following 2 small and 2 large doses in a vagotomized dog. E. Apneusis after the subsequent large dose in the same animal. F. Respiration 7 min. after the injection of 0.12 in a dog with intercollicular transection. G. Respiration following vagotomy performed in the same animal immediately after F. H. Mild apneusis after the injection of 0.12 in a vagotomized dog with intercollicular transection. I. Marked apneusis and respiratory failure following the subsequent injection of 0.12 in the same animal. J. In the first part of this tracing are shown apneustic respiratory movements after 1 small and 2 large doses in a dog with anterior pontile transection. After a 2 min. stop of the kymograph at X the third large dose was injected. K. Prolonged inspiration and decreased amplitude of respiration following the subsequent large dose given 2.5 min. after the injection indicated in J. L, M and N. Tracings showing the progress of respiratory failure in an intact dog after 4 large doses. O, P and Q. Tracings showing the progress of respiratory failure in a vagotomized dog after 1 small and 3 large doses.

300 per cent though the period of expiration showed no change (fig. 5G). In figure 5H and I are shown stages in the development of marked apneusis following repeated large doses in a dog with the vagi sectioned and the brain stem

transected in the intercollicular region. As sustained inspirations became more prolonged they were interrupted by shallow inspiratory movements which were followed by expirations (fig. 5I).

Apneustic respiration appeared after 3 injections of potassium phosphate in the dog with brain stem transection in the anterior pontile region though the vagi were intact (fig. 5J). This animal had received artificial respiration because breathing stopped after the brain stem was cut. Artificial respiration was discontinued when breathing reappeared. The first large dose after apneustic respiration was established induced a prolongation of inspiration (fig. 5J). The subsequent large dose was followed by a prolongation of the inspiratory phase and a diminution of the amplitude of respiration (fig. 5K).

The pattern of respiratory failure in the intact animal was formed by a gradual decrease in amplitude of the regular respiratory cycles and by the appearance of respiratory gasps at somewhat regular intervals (fig. 5L, M and N). These gasps were frequently superimposed upon a regular inspiration. Intermittent respiratory gasps were not characteristic of respiratory failure in vagotomized animals and when they were present they disappeared as respiratory failure progressed (fig. 5O, P and Q). The depth of respiration was gradually diminished and the inspiratory phase was prolonged. Respiratory failure occurred while blood pressure was still being maintained at normal or above normal levels.

Muscular twitching was observed when small doses were given. Large doses caused widespread rigidity with head retraction and strong extension of the limbs.

That the effects obtained in our experiments did not result from escape of the solution into the circulation was proved by the absence of the changes described above after the intravenous injection of amounts of potassium phosphate equal to those used intracisternally. Furthermore, equal volumes of Ringer's solution given intracisternally produced none of the effects obtained with potassium phosphate solution.

Intracisternal injection of $M/6 \text{ CaCl}_2$ in two dogs produced no immediate change in rate or depth of respiration. Ten to 15 minutes after the injection of 0.12 cc. per kgm., gradual diminution in the depth of respiration was followed by a moribund condition and cessation of breathing. No significant heart rate, blood pressure or electrocardiographic changes were observed. The slow heart rate which developed several minutes after the appearance of shallow respiration was perhaps due to a systemic effect of anoxia.

DISCUSSION. In attempting to analyze the effect on the sympathetic and parasympathetic activity of an increase in the K:Ca ratio in the cerebrospinal fluid it was found that the initial decrease in heart rate could be clarified by observing separately the effect of removing the sympathetic or parasympathetic outflow to the heart by cutting the spinal cord or the vagi, respectively. Downman and Mackenzie suggested that the transient slowing of the heart rate by intracisternal injection of potassium phosphate in the rabbit resulted from temporary depression of the sympathetic centers. That vagal stimulation cannot be ruled out in the dog is shown by the marked decrease in heart rate following

injections in animals with spinal cord section above the level of sympathetic outflow (fig. 1H and J). The marked bradycardia obtained by these injections contradicts the conclusion of Stern (1942) that intracisternal injection of potassium phosphate causes a depression of the parasympathetic center tone. Furthermore, the bradycardia which persists in the intact dog for several minutes following small doses (fig. 1D) disappears after bilateral vagotomy and the heart rate exceeds the pre-injection level (fig. 1E). Moreover, the increase in heart rate after large doses in the intact animals does not prove that the vagus center is depressed. The electrocardiographic records show the development of a tachycardia with a low pacemaker. The appearance and cessation of tachycardia in these animals are abrupt. It seems likely that rapid heart rate of *this type* can be obtained with simultaneous strong vagal and sympathetic stimulation.

The observation of a decrease in heart rate after intracisternal injections of KCl in sufficient quantity to produce a marked rise in blood pressure was not reported by Stern and Chvoles for the dog or by von Euler for the cat. The failure of bradycardia to appear in one of our animals suggests that the effect of our large doses was near a critical point where sympathetic stimulation gains dominance before parasympathetic effects can be observed. The failure of Stern and Chvoles and von Euler to obtain a slowing of the heart rate may have been due to a dosage above this critical level. In fact von Euler does report a marked bradycardia in cats receiving intra-arterial injections of KCl.

In vagotomized dogs given small doses the initial decrease in heart rate is not as marked as in the intact animals but a slowing of the heart rate did occur consistently in our experiments. The diminution of the extent of slowing in 2 vagotomized dogs and the absence of initial slowing in 3 other vagotomized dogs given large doses (fig. 2D) suggests that the decreased heart rate observed after small doses is not due to direct inhibition of the cardio-accelerator center. The similarity in temporal relationship of the initial bradycardia and the subsequent increase in heart rate in intact and vagotomized animals and in those with spinal cord section suggests that potassium phosphate first excited the cardio-inhibitory center with coordinate depression of the cardio-accelerator center and subsequently superimposed an opposite effect upon these centers. If it were assumed that the potassium produced its effect by diffusion into the nerve cells of the centers, an initial depressant effect on the cardio-accelerator center would have to be taken as an exception to the generally observed initial excitatory effect of potassium-treated nerve cells (Fenn, 1940, p. 398). However, if a reciprocal action between the centers is postulated the initial depression of the cardio-accelerator center may be attributed to excitation of inhibito-internuncial cells which impinge upon this center. The assumption that the potassium enters these cells simultaneously with its entrance into the cells of the cardio-inhibitory center would explain the simultaneous excitation and depression of the cardiac centers. If it is further assumed that cardio-accelerator cells and inhibito-internuncial cells which impinge upon the cardio-inhibitory center are excited simultaneously this would explain the subsequent simultane-

ous excitation of the cardio-accelerator center and depression of the cardio-inhibitory center.

No attempt was made by Resnik *et al.* to explain the initial fall in blood pressure following the intracisternal injection of small doses of KCl in the intact dog. From a comparison of the heart rate and blood pressure changes in the intact animals and in those with spinal cord section, it is evident that vasodilatation occurred in the former. Small doses produced a greater fall in blood pressure in the intact dogs though the decrease in heart rate was less. Whether the vasodilatation was due to a direct depression of the vasoconstrictor center by the potassium phosphate or due to a direct stimulation of a vasodilator center could not be determined under the conditions of these experiments, but again the assumption that K in small amounts stimulates nerve cells argues against the former action. Furthermore, an indirect depression of the vasoconstrictor center is suggested by the possibility that the potassium phosphate stimulates internuncial depressor nerve cells which carry impulses to the vasoconstrictor center.

All published reports examined agree that intracisternal injection of K produces a rise in blood pressure. The failure of most of these investigations to show a fall in blood pressure may have been due to a dosage not within the range of a critical level large enough to produce a depressor effect and small enough to avoid obliteration of the vasodilator effect by a pressor effect. In our experiments it was found that a gradual increase in the amount of potassium phosphate caused gradual displacement of the vasodilator effect by a pressor effect. When the dosage was large a marked increase in blood pressure, similar to that reported by Stern and Chvoles and by von Euler, followed immediately. Mullin, Hastings and Lees (1938) attributed a part of the rise in blood pressure to the marked increase in muscular tension. That the rise in blood pressure is produced primarily by stimulation of the vasoconstrictor center is shown by the maintenance of a somewhat lower but still high level after the tetanic seizure of the skeletal muscles has subsided. There are no experimental facts of which we are aware to support a suggestion that the simultaneous decrease in heart rate and increase in blood pressure which followed immediately after injection of large doses of potassium phosphate may be due to synchronous stimulation of the vasoconstrictor center and depression of the cardio-accelerator center. We interpret these results as evidence of simultaneous stimulation of the vagus and vasoconstrictor centers. Moreover, the assumption that the initial effect of large doses on the vasoconstrictor center is excitatory opposes the probability that the vasodilator effect of small doses is attributable to direct depression of this center. The more marked and prolonged rise in blood pressure in vagotomized animals was due in part to the separation of depressor fibers when the vagi were cut and in part to removal of efferent vagal influence on the heart. By contrast the marked slowing of the heart rate in intact dogs offers additional evidence that the parasympathetic centers are not paralyzed even by large doses of potassium phosphate.

In the above brief description of the electrocardiograms reproduced we have

not presented a detailed analysis of all the observed disturbances of cardiac rhythm and conduction. An extended discussion of the cardiac physiology concerned is not within the scope of this paper. We do, however, wish to emphasize the striking changes in cardiac activity which may be brought about by action at the medullary level upon both the vagus and sympathetic outflow to the heart. Failure to elicit disturbances in the rhythm and sequence of the cardiac cycles after cord section at C_6 demonstrates that the sympathetic cardiac nerves are primarily responsible for the more exotic changes observed in the intact animal. The vagal activity following injection of potassium phosphate causes a marked slowing of the heart rate without producing any gross disturbances of rhythm or conduction. If the vagi are left intact there is much less frequency of occurrence of such arrhythmias as those which we have described above, a fact at least consistent with the possibility that many of these arrhythmias appear to be of nodal origin.

Rothberger and Winterberg (1911) studied the effects of peripheral sympathetic stimulation and found that the right accelerator nerve increased the rate of the heart beat without changing the A-V sequence. The left accelerator increased the sinus rate but slightly, but in 30 per cent of their cases left accelerator nerve stimulation released A-V nodal rhythm. We have also observed that cardiac arrhythmias can be more consistently produced by stimulation of the left stellate ganglion. Beattie, Brow and Long (1930) reported production of extrasystoles by hypothalamic stimulation. Allen (1931) found that bigeminal rhythm could be produced in the rabbit heart by chemical stimulation of the trigeminal nerve following mesencephalic section of the brain stem. Thus the reflex which he described would be mediated over a path involving the trigeminal as afferent and having central connections entirely below and not involving the diencephalon. The failure of brain stem transection in the intercollicular or pontile region to interrupt the appearance of ventricular arrhythmias in our experiments proved that these effects are not dependent upon stimulation at the level of the hypothalamus.

It seems entirely possible that the cardiac arrhythmias which we have observed are due to stimulation of nerve elements lying in the floor of the fourth ventricle, causing excitation of pathways descending in the spinal cord and leaving in sympathetic nerves at the upper thoracic levels. That the action of the solution is not limited to the sympathetic outflow is shown by the marked vagal effect produced in animals with spinal cord section.

Using a heart-lung preparation of the dog, Anrep (1936) was able to demonstrate an effect of the sympathetic cardiac accelerators in producing respiratory arrhythmias. These arrhythmias appeared only after electrical stimulation of the peripheral end of the vagus. Under this condition he found that during strong inspirations the heart rate was somewhat faster than during the expirations. Anrep considered these respiratory arrhythmias to be of central origin and due to a reciprocal cooperation of inhibitory and accelerator cardiac fibers. The failure of potassium phosphate to produce marked cardiac arrhythmias of sinus origin in vagotomized dogs may have been due to acceleration of the heart

which followed section of the vagi. On the other hand, the ready production by potassium phosphate of ectopic beats and abnormal complex forms in coordination with the inspiratory phase of respiration in vagotomized dogs presents a condition in which the peripheral effect of accelerator center stimulation is enhanced by absence of vagal influence. These results suggest that the vagal fibers exert an effect on the heart antagonistic to the formation of cardiac abnormalities of nodal or perhaps even of ventricular origin.

The interpretations of previously reported effects of K on the respiratory center are contradictory. It is possible that some of the contradictions could be eliminated by examination of results in the light of observations made by Pitts, Magoun and Ranson (1939a) who found that in the cat faradic stimulation when applied to a localized region of the ventral reticular formation of the medulla immediately overlying the cephalic four-fifths of the inferior olive gave rise to maximal inspiratory reactions. Maximal expiration was obtained by stimulation of the dorsal reticular formation, dorsal to and slightly cephalic to the inspiratory reticular formation. In our experiment the initial interruption of inspiration and the displacement of the midposition of respiration toward the expiratory side suggest that a preponderance of expiratory stimulation is the first respiratory response to intracisternal injection of potassium phosphate. The failure of potassium phosphate injections to produce active expiration consistently is perhaps due to a widespread stimulation which would be expected to include some inspiratory reaction by the time any considerable expiratory response was effected. We have based our interpretation of the initial respiratory effects on the assumption that interruption of inspiration, whether this is followed by active expiration, passive expiratory apnea or reduced inspiration, is accomplished by stimulation of nerve cells which bring about expiratory reaction. The marked inspiratory movements which follow after a longer latent period are interpreted as being due to the eventual development of a preponderance of inspiratory stimulation. The inspiratory reactions were of such strength in some of our experiments that respiratory movements ceased for several seconds while marked inspiratory action was sustained. Similar inspiratory reactions were recorded by Hooker but interpreted as inhibition of the respiratory center.

If the above deductions concerning stimulation of the respiratory center are correct interpretations of the experimental facts we may assume that the nerve cells in the dog which bring about expiration are more quickly accessible to the potassium phosphate than those which control inspiration. The localization of a region near the surface of the medulla of the cat where faradic stimulation brought about maximal expiration (Pitts, Magoun and Ranson 1939a) supports this assumption. The sustained inspiratory reactions which follow the initial expiratory effects support the hypothesis set forth by Pitts, Magoun and Ranson (1939b) in which they assume that a strong stimulation of the inspiratory nerve cells tends through synaptic connection of these cells to set into activity simultaneously many neurons thus producing maximal inspiration. The restoration of expiration may be due to direct stimulation by the potassium phosphate of the

expiratory nerve cells plus the reflex excitation of expiration brought about by the strong inspiratory action. The increase in amplitude and rate of respiration which then follows is due to the increased level of excitability of the entire respiratory center.

The studies of Pitts, Magoun and Ranson (1939a) and Stella (1938) agree with the original observation of Marckwald (1887) that apneustic respiration is produced only when the vagi are cut and the brain stem is transected in the upper pontile level. In all of our experiments with vagotomized dogs in which sustained inspiratory reactions occurred the apneustic respiration interposed during the re-establishment of respiratory rhythm was regarded as evidence that strong stimulation of the inspiratory nerve cells was persisting. Most of these animals had not been subjected to brain stem section. In our vagotomized dogs, therefore, sustained inspiration appears to be due in part to release of the inspiratory mechanism from restraint which is normally exercised by afferent pulmonary fibers of the vagus and in part to a preponderance of inspiratory stimulation by potassium phosphate. The slightly apneustic respiration in two of the intact animals (fig. 4D and E) was produced entirely by a preponderance of inspiratory effect. The active expiration which accompanied this period of apneustic breathing in some animals (fig. 5C) suggests that the sustained inspiration was not due to depression of the expiratory activity by the potassium phosphate. Another line of evidence which supports the view that expiration is not being differentially depressed is found in the pattern of respiratory failure in intact dogs. Expiratory movement is prompt and complete throughout the progress of respiratory failure (fig. 5L, M and N). All animals dying in respiratory failure whether the inspiration is prolonged or brief suffer a decrease in the strength of inspiration.

That sustained inspiratory movements after potassium phosphate injections may be produced with the vagi intact is shown by the marked apneustic breathing in a dog decerebrated in the anterior pontile region (fig. 5J and K). In this animal it is probable that reduction of restraint on the nerve cells which bring about inspiration was effected through separation of the pneumotaxic center.

SUMMARY

1. The effects on blood pressure, heart rate, the cardiac cycle and the respiration of intracisternal injection of potassium phosphate have been studied.

2. Small doses (0.03 cc. of M/6 potassium phosphate per kgm.) produced an initial decrease in blood pressure followed by a gradual return to the pre-injection level. After vagotomy the amount of fall was diminished and the amount of rise in blood pressure was augmented. A prompt, marked rise in blood pressure occurred after large doses (0.12 cc. per kgm.). The pressor effect was exaggerated after vagotomy.

3. An initial decrease in heart rate which persisted was obtained after initial small doses were injected in intact animals. A transient bradycardia followed by a significant increase in heart rate was induced by initial small doses in vagotomized dogs. Large doses usually produced a decrease in heart rate fol-

lowed by a marked increase which did not persist in intact animals. A decrease in heart rate usually did not occur after large doses in vagotomized dogs and the rapid heart rate which followed was maintained for 15 minutes or longer.

4. Marked sinus arrhythmias which were frequently associated with the respiratory cycles were produced after injections in intact animals. Ectopic beats, frank tachycardias and a variety of complexes of abnormal form appeared after injections in vagotomized animals.

5. The first effect of potassium phosphate on respiration was an interruption of inspiration which was soon followed by marked stimulation of inspiration. Restoration of regular respiratory rhythm was accompanied by an increase in amplitude and rate of respiration.

6. Apneustic respiration usually followed injections in vagotomized dogs. Marked apneusis was observed after injection in an animal with the brain stem transected in the anterior pontile region.

7. Observations on heart rate and rhythm, blood pressure and respiration indicate that on the medullary centers potassium phosphate produces a direct stimulatory action which activates sympathetic, vagus and respiratory mechanisms.

8. Particular results of such action are discussed and explanations for their manifestation are suggested.

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THE FATE OF CO IN THE BODY DURING RECOVERY FROM MILD CARBON MONOXIDE POISONING IN MAN¹

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Fenn and his colleagues (1) have shown that carbon monoxide is oxidized to carbon dioxide by frog's muscle when this is exposed to atmospheres containing high percentages of CO, e.g., 80 per cent CO and 20 per cent O₂. The CO pressures met with in mammalian carbon monoxide poisoning are, however, only of the order of one-thousandth as great. Thus, in the case of man, prolonged breathing of air containing as little as 0.1 per cent CO is sufficient to raise the COHb saturation of the blood to the lethal limit of 60 per cent. In this much lower range of CO pressure, Haldane (2) has emphatically concluded both from his own work and that of others that "CO is not oxidized or otherwise decomposed in the human body of any living animal. . . . CO passes in by the lungs and passes out—far more rapidly than is generally supposed—by the lungs, without there being the smallest loss."

Evidence for this view, in the case of man, is given by the following type of experiment by Haldane and Loraine Smith (3): "The subject inhaled sufficient CO to saturate his blood to the extent of 33.3 per cent COHb. He then breathed for an hour into a small closed circuit, the CO₂ being removed and the O₂ replenished in the usual way. The volume of the closed circuit was so small that the amount of CO it could have removed from the blood in coming into equilibrium therewith must have been negligible compared with the total amount in the blood. Any drop in the per cent COHb during the hour of the experiment must accordingly have been due to non-expiratory loss of CO. Actually at the end of the experiment the COHb was found to have remained unchanged at 33.3 per cent."

This experiment is, however, open to the following comment. The experimental error of Haldane's method of COHb estimation is given as 2 per cent COHb (2), so that in the above experiment the blood might possibly have dropped from 33.3 to 31.3. Such an effect would be quite large in comparison with the total drop in per cent COHb found when a subject breathes ordinary air instead of into a closed circuit. Thus in one hour, our data (4) on the rate of elimination of CO from the blood of normal men at rest indicate that the blood COHb would only have dropped from 33.3 to about 28.0 per cent. Haldane's experiment therefore only seems to prove that the CO lost from the blood in non-expiratory forms cannot exceed $100 \times 2.0/5.3$ or 36 per cent of the total loss, but does not exclude lesser degrees.

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It accordingly seemed desirable to investigate the matter anew with the more accurate technique now available. In our first set of experiments we set out to make a balance sheet between the amount of CO lost from the blood and that appearing in the expired air during 15 minute periods within the first hour after the administration of carbon monoxide to normal man. The results and calculations reported below show that only about two-thirds of such loss of CO from the blood is accounted for in the expired air, the other one-third presumably disappearing by one or more of the following routes: (i) oxidation, or other forms of metabolism; (ii) diffusion through the skin or into the sweat, urine or feces; (iii) slow diffusion to and reversible combination with hemoglobin and/or similar iron-containing pigments situated outside the main blood stream. A further set of experiments indicated that practically the whole of the CO unaccounted for in the early period could ultimately be recovered in the expired air, if the subject continued to breathe oxygen for 4 hours. This result not only proves that factor (iii) is the chief one responsible for the early deficiency, but also substantiates Haldane's view of there being no appreciable loss of CO in the body, though the speed of exchange with the expired air is less than sometimes supposed. In an adjoining paper (7) it is shown, with the aid of radio-active CO, that less than 0.1 per cent of the CO lost from the blood during the first hour after administration of CO is oxidized to CO₂ in the case of normal men subjected to moderate doses of carbon monoxide, thus ruling out—in these instances—the attractive possibility raised again by Fenn's work on frog muscle. Some preliminary evidence is given in the same paper as to the sites in the body where the processes comprised under factor (iii) may occur, together with references to other work, relating to the fate of CO in the body.

I. THE RECOVERY OF CO IN THE EXPIRED AIR DURING THE FIRST HOUR AFTER ADMINISTRATION. The percentage of CO recovered in the expired air from the blood over any given interval t_1 to t_2 is equal to:

$$100 \times \frac{E_{1,2} c_{1,2}}{B(c_1 - c_2)} \quad (1)$$

where $E_{1,2}$ = volume of air expired during the interval

$c_{1,2}$ = average per cent CO in $E_{1,2}$

B = blood volume of subject

c_1, c_2 = CO contents of blood at t_1, t_2 respectively.

Of the quantities in equation (1) $E_{1,2}$ and B are easy to measure accurately, but the same is not true of $c_{1,2}$ or $(c_1 - c_2)$ since both of these are very small especially if the subject breathes air throughout the interval t_1 to t_2 . If oxygen is substituted for air, there is a 5 to 6-fold increase in the rate of CO elimination and thus also in the values of $c_{1,2}$ and $(c_1 - c_2)$, $E_{1,2}$ and B being practically unaffected. For this and other reasons the first tests were done with subjects breathing oxygen rather than air, the procedure in a typical experiment being as follows:

The subject breathes oxygen for 25 to 30 minutes through an oro-nasal mask. Towards the end of this period 10 cc. of his blood are drawn, and at the end of it 100 to 250 cc. of carbon monoxide are administered to him. A tonometer

containing the CO over mercury is connected by means of a short, tightly-fitting rubber tube with a number 20 hypodermic needle, which is thrust into the lumen of the rubber tube joining the oxygen cylinder to the mask. The CO is introduced over a period of 3 to 4 minutes; the subject usually absorbs about half of it, and the CO content of his blood increases by 1 to 2 volumes per cent. He continues to breathe oxygen through the mask for 15 minutes so as to permit the absorbed CO to become uniformly mixed throughout the circulation; he then holds his breath, quickly removes the mask and replaces it with a nose-clip and mouthpiece connected with the inlet and outlet rubber flutter valves. The inlet valve is connected to the oxygen supply and the outlet valve to a large rubber bag of 300 liters capacity, which had been previously washed out with oxygen. As soon as the nose-clip and mouthpiece have been adjusted, collection of the expired air begins and a blood sample (c_1) is simultaneously drawn without stasis from the antecubital vein, heparin being used as an anticoagulant. The expired air is collected for a measured time (15 to 30 min.), at the end of which a second blood sample (c_2) is drawn. The two blood samples c_1 , c_2 are analysed for CO by the combined syringe-Van Slyke method described by us (5); the difference between c_1 and c_2 is often only very small and, therefore, maximum analytical precision is required. The volume of gas expired is measured with a meter in the ordinary way; the per cent CO is determined with the aid of the subject's "blank" blood by means of the absorption method also described by us (6).

Numerical example of calculation of CO recovery in expired air:

$$c_1 = 1.09 \text{ volumes per cent}$$

$$c_2 = 0.85 \text{ volume per cent}$$

$$\text{Blood volume of subject, previously determined,} = 5700 \text{ cc.}$$

$$\text{CO lost from blood} = 5700 \times \frac{1.09 - 0.85}{100} = 13.7 \text{ cc. N.T.P.}$$

$$\text{Volume of expired air sample analysed for CO} = 240 \text{ cc.}$$

$$\text{CO found therein} = 0.0151 \text{ cc. N.T.P.}$$

$$\text{Total volume of expired air} = 147,100 \text{ cc.}$$

$$\text{Therefore, total CO expired} = \frac{147,100}{240} \times 0.0151 = 9.26 \text{ cc. N.T.P.}$$

$$\begin{aligned} \text{Total volume of CO inspired from oxygen cylinder (CO content} &= 0.00066 \\ \text{per cent)} &= 147,100 \times \frac{0.00066}{100} \times 0.9 = 0.87 \text{ cc. N.T.P.} \end{aligned}$$

(0.9 = the correction factor for temperature and pressure)

$$\begin{aligned} \text{Total volume of CO contributed by the body to the expired air} &= 9.26 \\ &- 0.87 = 8.39 \text{ cc. N.T.P.} \end{aligned}$$

$$\begin{aligned} \text{The proportion of CO lost from the blood which is recovered in the expired} \\ \text{air is thus equal to } 100 \times \frac{8.39}{13.7} = 61.2 \text{ per cent.} \end{aligned}$$

The overall accuracy of the recovery figure depends, of course, on the accuracy of the individual quantities in equation (1). Of these, $E_{1,2}$ can be depended on to ± 1 per cent of itself, $e_{1,2}$ to ± 2 per cent of itself and $c_1 - c_2$ to about ± 10 per cent of itself. The value of the blood volume is probably correct to ± 5 per

cent of itself, whether it be measured by the carbon monoxide or the dye methods which tally well with each other, according to recent investigations (4).

Table 1 shows the results of such experiments on three subjects. In each case the recovery is close to 60 per cent and is independent of the blood CO content over the range of 1 to 2 volumes per cent. In three more recent experiments in which the subjects absorbed CO containing traces of the radio-active gas (7) the same average recovery of 60 per cent was obtained, though the scatter was much bigger than in the results of table 1.

A similar set of experiments was next done at a partial pressure of oxygen corresponding to that in air at sea level. The first three of these (see table 2) were carried out in the decompression chamber of the Aviation Research Laboratory, Welfare Island, New York City, with the subjects breathing 99 per cent O₂ at a simulated altitude of 35,000 feet. The total pressure was 180 mm. Hg and the alveolar partial pressure of O₂ was thus about 100 mm. Hg, i.e., the normal value in air at sea level. There is no reason to suppose that the lowered pressure

TABLE 1
Recovery of CO in expired air of men breathing oxygen at sea level

SUBJECT	EXPIRED AIR		CO EXPIRED	c ₁	c ₂	BLOOD VOLUME	CO LOST FROM BLOOD	PER CENT RECOVERY IN EXPIRED AIR
	Time of collection	Volume						
	min.	liters	cc. N.T.P.	vols. per cent	vols. per cent	cc.	cc. N.T.P.	
R. P. N.....	15	147	8.25	1.09	0.85	5,700	13.6	61
W. W. W.....	15	144	18.9	2.03	1.51	5,700	30.2	63
J. W.....	18	150	12.2	1.60 ₅	1.23 ₄	5,750	21.3	57
Mean.....								60

per se has any effect on the rate of disappearance of CO from the blood of man, any more than it has on the rate of CO uptake (8), provided care is taken to avoid "bends"; with the practical exclusion of nitrogen from the expired air, however, the accuracy of our method of estimating CO (6) can be much enhanced. At the time of these experiments (15 months ago) the accuracy of our estimation of low percentages of CO in ordinary air was scarcely good enough, and it was for this reason that these low pressure experiments were undertaken. We are greatly indebted not only to Dr. A. Barach for placing the necessary facilities at our disposal, but also to Mr. M. Eckman and his colleagues both for indoctrinating us and our subjects in the use of the chamber and for their skilled co-operation in the experiments. The procedure adopted was substantially the same as before, save that the subjects (and experimenters) were preoxygenated for one hour before the decompression started in order to reduce the incidence of "bends." The CO was administered in the latter part of the preoxygenation period, at the end of which the pressure in the chamber was reduced over a period of 20 minutes to 180 mm. Hg, i.e., to that corresponding to 35,000 feet

altitude. The first blood sample, c_1 , was taken 2 or 3 minutes after arriving at "35,000 feet"; the second sample, c_2 , was taken 15 to 30 minutes later. Both these samples were analyzed outside the chamber. The volume of the expired air, V cc., was measured in the chamber at 35,000 feet by means of a spirometer, and a sample transferred into a 550 cc. tonometer also in the chamber. The amount of CO in the tonometer was determined after removal from the chamber, and the total amount expired is calculated therefrom by multiplying by $V/550$.

The results of the chamber experiments on the same 3 subjects as were used in table 1 are given in table 2. The experiment on W. W. W., with a per cent recovery of only 40, was the first to be performed and was not entirely satisfactory from a technical standpoint; in the other two experiments, especially that on R.P.N., the technical conditions were quite good. The per cent recoveries in these two cases, namely 65 and 66.5, are close to those previously found but, owing to the smallness of $c_1 - c_2$ (0.10 volume per cent), they are subject to a much greater uncertainty than are the values in table 1.

Table 3 gives the results of two recent experiments on subjects breathing air at sea level, with longer periods of expired air collection and with the methods of analysis at their maximal accuracy, viz., 0.0001 to 0.0002 per cent atmospheres in the case of the CO of the expired air and 0.01 to 0.02 volume per cent in the case of the blood CO content. In the first test, on W. W. W., the recovery during the first of the two half-hour periods, i.e., 72 per cent, agrees within experimental error with the previous range of 60 to 65 per cent, in view of the smallness of $c_1 - c_2$, which in the present instance is only about 5 times the experimental error of the determination. The figure for the second half hour period is higher, at 82 per cent; this may be, in part, due to experimental uncertainty but, in part, also to a real rise in the percentage recovery in the later stages (see below). The figure of 78 per cent recovery obtained in the case of R.P.N. may also be genuinely higher than the previous range, but in this instance the CO content of the blood was raised to twice the usual value, i.e., 4 volumes per cent as compared with 2 volumes per cent. Further tests are clearly required at these higher blood CO contents.

The results so far, however, seem definitely to indicate that the expired air only accounts for 60 to 70 per cent of the CO lost from the blood during the first hour following the rise of blood CO content to ca. 2 volumes per cent. The reasonableness of this conclusion is also confirmed by the theoretical calculation given in the Discussion. A similar result was obtained in cruder experiments listed in table 4 upon the per cent recovery of CO in the expired air of unanesthetized dogs in hemorrhagic and traumatic shock. These were our first experiments on the per cent recovery of CO, having been done 18 months ago. The dogs breathed ordinary air through an intratracheal cannula, and the expired air was collected and analysed in the usual way. The rate of loss of CO from the blood is seen to be markedly greater than that from normal men breathing ordinary air; the difference is due not so much to the change in species as to the fact that in these conditions of shock the pH of the blood is more acid than normal and also there is marked hyperventilation. The estimates of the CO

lost from the blood are, owing to the relatively large values of $c_1 - c_2$, fairly reliable; the percentages of CO in the expired air are on the other hand so small (i.e., of the order of 0.001 to 0.003 per cent) that, with the gas analysis technique in its early form, it may well be that the estimates of the CO eliminated in the

TABLE 2

Recovery of CO in expired air of men breathing 99 per cent oxygen at simulated altitudes of 35,000 feet (pressure = 180 mm. Hg)

SUBJECT	EXPIRED AIR		CO EXPIRED	c_1	c_2	BLOOD VOLUME	CO LOST FROM BLOOD	PERCENT RECOVERY IN EXPIRED AIR
	Time of collection	Volume at ambient pressure						
	min.	liters	cc. N.T.P.	vols. per cent	vols. per cent	cc.	cc. N.T.P.	
R. P. N.....	22	163.6	2.98	1.54	1.46	5,700	4.56	65
W. W. W.....	15	101.8	2.05	1.63	1.54	5,700	5.13	40
J. W.....	27	203.3	3.83	1.56	1.46	5,750	5.75	66.5

TABLE 3

Recovery of CO in expired air of men breathing air at sea level

SUBJECT	EXPIRED AIR		CO EXPIRED	c_1	c_2	CO LOST FROM BLOOD	PERCENT RECOVERY IN EXPIRED AIR
	Time of collection	Volume					
	min.	liters N.T.P.	cc. N.T.P.	vols. per cent	vols. per cent	cc. N.T.P.	
W. W. W.....	10 to 40	215	4.50	1.65	1.54	6.27	72
	100 to 130	213.3	4.35	1.36	1.27	5.42	82
R. P. N.....	10 to 40	226	12.0	4.19	3.92	15.4	78

TABLE 4

Recovery of CO in expired air of dogs in hemorrhagic and traumatic shock

CONDITION OF DOG	EXPIRED AIR		CO EXPIRED	c_1	c_2	BLOOD VOLUME	CO LOST FROM BLOOD	PERCENT RECOVERY IN EXPIRED AIR
	Time of collection	Volume						
	min.	liters	cc. N.T.P.	vols. per cent	vols. per cent	cc.	cc. N.T.P.	
Trauma.....	30	140	1.32	1.30	0.72	528	3.06	43
	25	82	1.64	2.68	1.73	567	5.38	30
	30	127	1.24	0.89	0.57	577	1.85	67
Hemorrhage.....	10	90	1.30	2.00	1.20	523	4.18	31
	30	200	0.82	0.90	0.65	616	1.54	53

expired air were only accurate to ± 30 per cent of themselves. Even so, it is clear that the per cent recovery of CO in the shocked dogs was only of the order of 50 per cent or less.

II. THE RECOVERY OF CO IN THE EXPIRED AIR DURING FOUR HOURS OF OXYGEN BREATHING. If the fraction of CO, which escapes from the blood but is unac-

counted for during the first hour after administration, is all reversibly combined with substances outside the main blood stream, it should ultimately dissociate therefrom and be completely recovered in the expired air if the latter be collected for long enough. If ordinary air is breathed, the half-time of CO elimination from the body is about 4 hours and the expired air would thus need to be collected for 24 hours which would be an impractically long time. In the case of oxygen, however, the half-time is shortened to about 40 minutes so that 4 hours collection of expired air should be enough to recover at least 98 per cent of the administered CO, provided that the amounts lost by oxidation (and/or other irreversible chemical reactions) and by diffusion via the skin, urine, and feces are really negligible. It therefore seemed crucial to carry out such experiments with prolonged oxygen breathing, and the general plan we adopted was accordingly as follows:

The subject (a non-smoker) is first made to absorb an accurately measured volume of CO—of the order of 150 cc.—and is then given oxygen to breathe for a period of 4 hours during which the whole of his expired air is collected. Its volume and average CO content are measured, their product being equal to the total volume of CO expired. Division of the latter by the volume of CO absorbed gives the percentage of CO recovered in the expired air, if a correction (small in the case of non-smokers) be applied for the difference in the respective CO contents of the blood at the beginning and at the end of the experiment.

Detailed procedure. (a) The subject reclines for at least half an hour and a 10 cc. sample of his antecubital venous blood is then drawn without stasis, heparin being used as anticoagulant. The CO content is subsequently estimated to within 0.01 to 0.02 volume per cent by our recently described method (5).

(b) The resting subject is next fitted with a leak-tight oxygen mask, connected on its intake side through an inspiratory valve to an anesthetic bag of 4 liters capacity, a supply of compressed air and a tonometer of 300 to 350 cc. volume containing CO of analyzed purity prepared from formic and sulfuric acids. The exit tube of the mask is connected through an expiratory valve to a large rubber bag, A, of 200 to 300 liters capacity. The CO is all displaced by 30 per cent NaCl into the intake feed of air over a period of 3 minutes, and the anesthetic bag inflated and deflated at least 5 times during 7 further minutes of air breathing so as to ensure that all the CO is washed into the mask. The expired air is collected over the whole 10 minute period, at the end of which the subject is at once switched from air to oxygen and the expired gas collected in a second large rubber bag, B.

(c) The expired air in the first bag, A, is meanwhile mixed by thorough beating, its volume measured in a Tissot spirometer, and a 500 cc. sample of it set aside in a tonometer for subsequent estimation of the CO percentage (6). The volume of CO absorbed by the subject is given by the difference between that contained in the tonometer and the total amount found in the first rubber bag. The latter is washed out several times with CO-free air and is then ready to replace the second bag, B, when the latter has been filled by the subject.

(d) The subject's expired gas is then turned to the first bag, during which the

second bag is treated in the following way: It is first thoroughly mixed and then connected to a graduated cylinder filled with 30 per cent NaCl and joined at its base to a large leveling bulb. After the connections have been flushed out with the expired gas, about 300 cc. of the latter are drawn into the cylinder. The volume of the remaining gas is then measured in the Tissot spirometer, and exactly one-thousandth of the amount so found is transferred from the graduated cylinder to an evacuated bottle of 2500 cc. capacity. The second bag is then ready to replace the first bag when this has been filled.

(e) Whilst the subject's expired gas is being collected in the second bag, the first bag is measured and one-thousandth of its volume transferred to the evacuated bottle as in (d). The same alternating procedure is continued throughout the 4 hours of oxygen inhalation, so that at the end the whole volume of gas expired during the period has been collected and gas of the average CO content for the whole period collected in the bottle.

(f) The mixed samples of gas in the bottle are brought to atmospheric pressure by admission of tap water and displaced thereby into a 500 cc. evacuated tonometer having taps at either end. The O_2 and CO_2 therein are next removed by 5 minutes' violent shaking with Krogh-Fieser solution (16 grams $Na_2S_2O_4$ + 14 grams. KOH + 2 grams Na-anthraquinone β sulfonate = 100 grams water) and the residual gas ($CO + N_2$) brought to atmospheric pressure by admission of tap water and then quantitatively displaced, by forcing mercury through the bottom tap of the tonometer into a modified Hempel pipette (9) full of mercury. Finally the gas is quantitatively transferred from the Hempel pipette to the chamber of the Van Slyke-Neill gasometric apparatus and its CO content determined by shaking with blood solution, according to our recent method (6).

A much simplified technique would have been possible if the whole of the expired gas could have been collected in a single container. The procedure may be further illustrated by going through the numerical results and calculations in one of our experiments.

Subject E. M.

Inspiration of CO: volume of tonometer = 325 cc.; purity of CO = 97.8 per cent; correction factor for temperature, barometer and aqueous vapor pressure = 0.886; volume of CO administered = $325 \times 0.978 \times 0.886$ = 281.6 cc. N.T.P.

Expiration of CO during 10 minute period: volume of gas expired = 75.1 liters as measured by spirometer = 75.1×0.886 = 66.5 liters N.T.P.; percentage of CO in expired gas = 0.23 per cent; volume of CO unabsorbed = $66,500 \times 0.23/100$ = 153 cc. N.T.P.

Volume of CO absorbed = $281.6 - 153$ = 128.6 cc. N.T.P.

Total volume of gas expired over 4 hour period = 1101.1 liters as measured by spirometer; = 1101.1×0.886 = 976 N.T.P.

Percentage of CO in average expired gas of 4 hour period = 0.0121 per cent.

Percentage of CO in inspired oxygen gas—none detectable.

Total volume of CO expired during 4 hour period = $976,000 \times 0.0121/100$ = 118.2 cc. N.T.P.

Blood CO content at end of 4 hour period = 0.23 volume per cent.

Blood CO content before administration of CO = 0.08 volume per cent.

Blood volume = 4.5 liters.

Total extra CO in blood at end of 4 hour period = $4,500 \times (0.23 - 0.08)/100$ = 6.75 cc. N.T.P.

Percentage recovery of CO in expired air over 4 hour period = $100 \times 118.2 / (128.6 - 6.75)$
 = 96.9 per cent.

In two other similar experiments (subjects F.T., H.S.) the percentage recoveries obtained were 93 and 97 respectively, the average of all three being 95.6. In view of the experimental errors of the CO determinations and the possibility of slight leaks in the masks, rubber bags, processes of transfer, etc., so nearly complete recovery seems as good as could have been expected, and indicates that practically all of the CO absorbed by the body must have been *reversibly* combined with the hemoglobin of the blood and similar compounds in the cells. The results are in marked contrast to those obtained by similar analytical procedures during the first hour of CO elimination, though it is true that the figure for the blood volume is important in the determinations over the first hour and relatively insignificant in the four hour experiments. The blood volume figure would have to be 50 per cent too high, if our finding of 60 to 70 per cent recovery in the first hour were to be explained by experimental error. Errors of such a size in the blood volume are most unlikely and, all in all, the results seem to indicate conclusively that practically the whole of the CO lost from the blood, and unaccounted for in the expired air during that period, must have diffused to and combined slowly, but reversibly, with compounds outside the main stream of the circulation.

The three experiments just quoted were done in the Aero-Medical Laboratory of the Donner Institute, University of California, Berkeley. We are greatly indebted to Dr. J. H. Lawrence for his interest and provision of facilities, to Dr. C. Tobias for his indefatigable aid in the actual experiments, and to the three volunteer subjects.

DISCUSSION. Since only 60 to 70 per cent of the CO lost from the blood during the first hour is found in the expired air, whereas virtually complete recovery is obtained if oxygen breathing is continued for 4 hours, it follows that during the later phases the CO lost from the blood must be *less* than that currently found in the expired air, the balance being made up by the CO which dissociates from combination with substances outside the blood stream and diffuses into the latter. In many CO blood volume determinations on normal men we have followed the fall in blood CO content with time, and found that with the subjects at rest and breathing ordinary air the blood CO content, when plotted logarithmically against time after the CO administration, gives a straight line for one hour provided that the earliest point is at 15 minutes or more after the end of the CO administration. We have only a few observations beyond one hour, but these tend to show a lesser rate of fall as would be expected from the consideration just mentioned. More accurate studies in this extended range would be of interest, especially in regard to the question of whether the blood CO content can be reduced to a negligible amount by breathing oxygen long enough; if it cannot, new light might be shed on the old, but still unsettled, suggestion raised by the French school (10), namely, that the body steadily produces small amounts of CO by endogenous metabolism.

Theoretical calculation of the percentage recovery of CO in the expired air during

the first hour after administration. As mentioned in the Introduction, the results of such calculations for the case of normal men breathing air confirm the previous experimental finding of 60 to 70 per cent recovery, as the following numerical example shows:

Let the blood CO content at zero time be 2.0 volumes per cent.

After 60 minutes the CO content, according to the average of the data given in table 2, is 1.68 volumes per cent.

The corresponding percentages of COHb are 10 and 8.4 respectively, and the average over the one hour period 9.2.

The average arterial pCO (partial pressure of dissolved CO) for the hour period

$$= \frac{\text{arterial } pO_2 \times \text{average arterial per cent COHb for the hour period}}{210 \times \text{average arterial per cent } O_2\text{Hb for the hour period}}$$

according to the equilibrium data of Sendroy, Liu, and Van Slyke (11) at 37°C.

Taking the arterial pO_2 at sea level as 100 mm. (12, 13), the average arterial per cent COHb as 9.2, and average arterial per cent $O_2\text{Hb}$ as 88.5, it follows that the average arterial pCO = $(100 \times 9.2)/(210 \times 88.5) = 0.05$ mm. Hg.

Now the average alveolar pCO for the hour period must be less than the average arterial pCO, by an amount equal to that required to drive the CO across the lung membrane by diffusion.

With a blood volume of 5700 cc. (the average in tables 1 and 2) the CO lost from the blood per minute in the present instance = $5700 \times (2.0 - 1.68)/(100 \times 60) = 0.304$ cc. N.T.P.

With an average lung Diffusion Constant of 30 (14), the arterial pCO minus the alveolar pCO must therefore be $0.304/30 = 0.01$ mm. Hg.

The average alveolar pCO thus = $0.05 - 0.01 = 0.04$ mm. Hg.

The average ventilation rate of the 5 subjects at rest is 6.5 liters (N.T.P.) per minute, and the dead space of the lungs divided by the tidal air at rest may be assumed to average 0.3.

Thus during rest the subject expires $6.5 \times 0.3 = 1.95$ liters of dead space air per minute containing no CO furnished by the blood, and $6.5 \times 0.7 = 4.55$ liters of alveolar air per minute containing a partial pCO = 0.04 mm. Hg furnished by the CO of the blood.

The total CO lost into the expired air per minute from the blood thus = $4550 \times \frac{0.04}{760} \times \frac{273}{310} = 0.212$ cc. N.T.P.

The average recovery of CO in the expired air during the one hour period is accordingly $100 \times \frac{0.212}{0.304} = 70$ per cent (\pm ca. 12 per cent), i.e., a figure in the same range as that found experimentally.

Owing to the approximate character of some of the numerical assumptions involved, this calculation is probably only accurate to about 1 in 6 of itself, and a possible error of ± 12 per cent is therefore attached.

In addition to these numerical uncertainties, there is a theoretical source of

error to be mentioned. The arterial pCO with which the blood leaves the lung capillaries is considerably higher than the pCO in the mixed venous blood entering the capillaries; in the above example the mixed venous pCO works out at about 0.025 mm. Hg as compared with an arterial pCO of 0.05 mm. Hg. At an alveolar pCO of 0.04 mm. Hg some CO would therefore be absorbed by the blood at the venous end of the lung capillaries; the actual alveolar pCO must accordingly be lower than 0.04 mm. Hg, but by how much is difficult to calculate. An overestimate of the alveolar pCO would in turn make the per cent recovery of 70 proportionately too high; unless and until some systematic error in the opposite direction comes to light, we feel reasonably safe in believing that the calculated figure of 70 ± 12 per cent recovery is, if anything, too great.

The extra-circulatory, reversible combination of CO. Two types of reversible combination outside the main blood stream suggest themselves: (a) chemical reaction with intracellular hemoglobin-like pigments, e.g., the myoglobin of the red muscles; (b) slow diffusion to and reaction with the hemoglobin of stagnant red cells in, e.g., the spleen, skin or bone marrow.

The proportion of CO combined with the myoglobin in man is only estimated to be about 5 per cent (15) of that combined with the hemoglobin of the circulating red cells, even when the distribution of CO in the body has reached complete equilibrium. The meager share secured by the myoglobin is in part due to its lesser quantity in the body and in part to its much lower affinity for CO, as compared with the blood hemoglobin. It is difficult therefore to see how anything like so large a fraction, as 30 to 40 per cent, of the CO lost from the blood in the first hour could be combined with myoglobin. Furthermore, according to recent theoretical work by one of us (16), it is probable that the actual rate of distribution of CO from the red cells to the myoglobin is far faster than generally supposed, for the calculated time of half reaction is only about of the order of 10 seconds, which is short compared with the tempo of the slow processes occurring in the first hour. For this reason also the significance of the rôle of the myoglobin in the latter is very doubtful.

Recent experiments on the distribution of radio-active CO in normal men (7) have suggested the possible presence, in the liver, of extra-circulatory substances with a much higher affinity for CO than blood hemoglobin has. Such substances may be closely related to, or identical with, the pseudohemoglobin of Barkan (17), which he regards as an intermediate in the breakdown of hemoglobin to bile pigments and which has likewise been postulated to have a much higher affinity for CO. Further work is required with radio-active technique on normal men and dogs; in the latter direct extracts of the liver, as free as possible from blood, should be studied in regard to their CO affinity.

The possible rôle of stagnant cells in the spleen, skin, etc., is obviously suggested by Barcroft's work (18). In man, however, there is, according to more recent investigations, very little increase in the total volume of circulating red cells when he passes from rest to hard work; this makes it very doubtful whether the storage of red cells in stagnant areas is, in man, nearly so significant a factor as Barcroft's work proved it to be in the case of dogs and cats, especially as

regards the spleen. The total amount of hemoglobin in the bone marrow in man is not known to us, but estimates for dog (19), namely 1 to 3 per cent of the circulating blood hemoglobin, make it unlikely to be an important factor.

The whole problem is therefore open for much further study, which might indeed throw new light not only on the chemical physiology of CO on the body but also on blood volume problems. The present work does, indeed, already raise a point in regard to the determinations of blood volume by the CO method. In the usual technique a known volume of CO is absorbed from a small closed respiratory circuit, and the plateau blood CO content, which is reached after 15 to 20 minutes of continued breathing in and out of the closed circuit, is used for the calculation of the blood volume. During such period, however, CO should be steadily diffusing out of the blood stream into reversible combination outside. The proportion so lost should be about one-third of the total CO lost from the blood, when the subject breathes air on open circuit for 20 minutes, i.e., one-third of 5 per cent, i.e., 2 per cent. Such an effect would be probably too slight to detect by repeated blood CO determinations in the plateau period, but would, nevertheless, cause the calculated blood volume to be systematically too high by 2 per cent. In the modified method used by us during the past two years (4), this small possible error is eliminated.

When man is exposed to an atmosphere containing a low percentage of CO, it is theoretically impossible for true equilibrium to be reached between the CO in the alveolar air and the blood, as long as CO is being steadily lost from the blood stream to extra-circulatory substances. In place thereof there should result a steady state, wherein the arterial $p\text{CO}$ is lower than the alveolar $p\text{CO}$ by such an amount that the rate at which CO steadily diffuses into the blood from the lungs is equal to that at which it is lost from the blood to other sites. Calculations, based on the data of this paper, show, however, that the differences between such steady states and the true equilibrium are too small to be of practical importance, when breathing ordinary air or O_2 at sea level.

It should be emphasized again that the present experiments have been limited to very mild amounts of CO poisoning in man at rest and with adequate O_2 supply. It would be clearly desirable to extend them to more marked degrees of CO-anoxia, to hypoxic anoxia, to exercise, to shock and other pathological conditions, and to the problem of CO-acclimatization, if the marked instances of this which Killick (15) has described can be reproduced. It seems possible that our findings may have some bearing on the experimental results which led Haldane and his co-workers (2) to their claim of oxygen secretion by man at high altitudes, since their method of estimating the arterial O_2 tension depended on the existence of an equilibrium between the pressure of CO in the alveoli and the blood rather than on the maintenance of a steady state.

Further notes on the question of irreversible loss of CO by non-expiratory channels. Our results not only run counter to the possibility of CO metabolism in the body, but also to significant loss by diffusion either into the air through the skin, or into the sweat, urine, and feces. The lack of loss through the skin is entirely in agreement with the work of Behnke and Willmon (20), who found that only

about 20 cc. of nitrogen diffused through the skin of normal men per hour under a pressure gradient of 600 mm. Hg. In the experiments of tables 1, 2, and 3 the gradient of pCO from the blood to the air could not have exceeded 0.2 mm. Hg at most, which, since the diffusion coefficients of CO and N₂ are about the same, would cause a diffusion of about 0.007 cc. CO per hour from the blood to the air through the skin. This figure is only of the order of one-thousandth the observed rate of CO loss from the blood.

The actual volumes of sweat, urine, and feces in man are small compared to his total volume, and, unless these media contain large amounts of substances with a remarkably high CO-combining power, it would seem impossible for them to remove significant amounts of CO.

Virtual exclusion of CO metabolism in the body applies not only to its oxidation to CO₂ but also to its hydration to formic acid and formate ions, i.e.:



From the data of Branch (21), to which Dr. D. Rittenberg kindly called our attention, it can be calculated that, at equilibrium 37°C, the following equations hold good:

$$(\text{CO in atmospheres})/2.0 = [\text{HCOOH}] = [\text{H}^+][\text{HCOO}']/1.8 \times 10^{-4}$$

With 2.0 volumes per cent CO in the blood, the corresponding pCO is about 7×10^{-5} atmospheres and the theoretical equilibrium concentration of formate ions at pH 7.0 works out at 0.06M. Thermodynamically it is therefore possible for the CO of the blood to be hydrated almost completely to formate, which might be further metabolized. Actually, however, the rate of hydration of CO to formate is immeasurably slow in absence of catalysts, and would only become significant in the body if a special enzyme were present for this purpose. Such an enzyme, if it existed, would be analogous to carbonic anhydrase, which catalyzes a rather similar chemical reaction, namely, the hydration of CO₂ to H₂CO₃ and HCO₃'.

Oxidation, hydration, or other metabolic reactions of CO, if they occur at all in the body, must presumably do so outside the blood in view of the remarkable stability of COHb in drawn blood. We have confirmed this stability in a recent experiment on human blood treated with CO and then equilibrated with an equal volume of oxygen gas and stored for 18 hours at 37°C. These conditions should be favorable for loss of CO by oxidation or other metabolic reactions. The CO content of the blood in this experiment was found, by our most accurate technique (5), to be 2.59 volumes per cent initially and 2.58 volumes per cent at the end of the 18 hours—an insignificant difference. This experiment clearly indicates that there is no enzyme in the red cells capable of oxidizing CO to CO₂ and furthermore that the abundant carbonic anhydrase of the red cells has no "formic anhydrase" properties.

SUMMARY

1. Analyses are given of the CO content of expired air and of the progressive fall in CO content of the blood during recovery from mild CO poisoning. From

such figures, together with the blood volume, the percentage of CO recovered in the expired air is calculated.

2. In normal men, whether breathing oxygen or air, the CO found in the expired air only averages 60 to 70 per cent of that currently lost from the blood during the first hour after the CO administration. Independent theoretical calculations confirm the validity of this result.

3. If, however, the subjects continue to breathe oxygen for 4 hours after the CO administration, about 96 per cent of the CO initially absorbed by the subject is recovered in the expired air over this longer period. It is inferred that the 30 to 40 per cent fraction of CO, lost from the blood during the first hour and unaccounted for in the expired air, must have combined reversibly with hemoglobin-like pigments outside the main blood stream. Subsequently as the blood CO content falls, the CO dissociates reversibly from these combinations, diffuses back into the blood and thence into the expired air.

4. The results of the four hour experiments confirm previous views that there is no significant loss of CO (a) through the skin, sweat, urine, or feces, (b) by oxidation or other forms of metabolism—at all events in mild CO poisoning and with the body adequately supplied with oxygen.

5. The possible sites for this extra-circulatory combination of CO are considered and its implications in regard to blood volume determinations and other aspects of the chemical physiology of CO are discussed.

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THE ELIMINATION OF CARBON MONOXIDE FROM THE HUMAN BODY WITH REFERENCE TO THE POSSIBLE CONVERSION OF CO TO CO₂

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The effect of carbon monoxide upon respiratory metabolism has been the subject of long controversy: Is the only effect of CO that of combination with hemoglobin, thus depriving the blood of some of its oxygen, or does CO react in some additional manner in the body? Is there any possible conversion of CO into CO₂ or other compound, or is there any other way in which the body can take up and store CO?

In addition to their theoretical implications, these questions are of practical importance, both in industrial and in military problems. Thus in the present war, in certain types of planes, tanks, cargo ships and in other situations, many fighting men may be exposed to low concentrations of CO for comparatively long periods of time and may suffer from carbon monoxide poisoning of a more or less severe degree. Therefore, the questions outlined above carry added significance.

In the past, considerable work has been done in this field, but there is only space here to refer to some of the more important observations. J. S. Haldane (1) exposed mice to a gas mixture containing 1 atmosphere of carbon monoxide and 2 atmospheres of oxygen. Under these conditions the hemoglobin of the blood was almost entirely combined with CO but enough oxygen was dissolved in the blood to maintain life. The behavior of the mice was practically normal, and Haldane thus concluded that CO was entirely inert except for its power to combine with hemoglobin. One year later Haldane and Smith (2) showed that when a very low pressure of carbon monoxide in a rubber bladder is in equilibrium with that in the human body in a closed respiratory system so that no CO can escape, the percentage of COHb in the blood remains the same (33 per cent) for as long as 60 minutes. This result was interpreted as ruling out metabolism of CO or slow accumulation of CO in any organ (e.g., the spleen) for then the blood COHb level would decrease progressively, and the concentration of CO in the bladder would fall off. Such a conclusion is, however, open to criticism (see 9).

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Since these experiments were done, the measuring techniques have been greatly improved, and the study of the effects of high concentrations of CO has been considerably advanced. In 1926 Warburg (3) showed that CO combines with a respiratory ferment in yeast and inhibits respiration. J. B. S. Haldane presented some evidence (4) pointing to a toxic effect of CO on rats when exposed to a mixture of 2 atmospheres of CO with 2 atmospheres of O₂. In 1932 Fenn and Cobb (5) observed that CO has a stimulating effect on the normal resting metabolism of frog and rat muscle (skeletal and cardiac) when exposed to gas mixtures containing high percentages of CO; e.g., 80 per cent CO and 20 per cent O₂. This was confirmed by Schmitt and Scott (6), who also drew attention to concurrent inhibition of oxidation in the presence of CO. These phenomena have been analyzed more recently by Stannard (7), who definitely concludes that the effect of CO on muscle is dual, consisting under certain conditions of an inhibition of the Warburg-Keilin cytochrome oxidase system together with a catalytic oxidation of CO to CO₂ by enzymes separable from the known resting and active systems. In the latter connection he quotes Negelein's demonstration (8) that CO can be burned to CO₂ in alkaline solution by green and "mischfarbene" hemins at low O₂ pressures.

No information has, however, been available on the magnitude of any possible "tissue" effects of CO when administered in very low concentrations (up to 0.01 per cent in the inspired air), so two of us recently (9) tried to recover CO, previously absorbed by man, by continued breathing of oxygen or air. The blood concentrations of COHb were obtained after venepuncture. The total amount of CO in the blood was estimated from the known blood volume and COHb concentration, and the CO content of all the exhaled air was also determined. The tests were continued for one hour after the absorption of CO and, in general, about 30 to 40 per cent of the amount of CO disappearing from the blood did not appear in the exhaled air. It seemed reasonable to suppose that some of the missing CO might have been oxidized to CO₂, since the previous evidence against this happening to a slight extent in man did not appear to us conclusive, and furthermore a calculation kindly made for us by Dr. W. O. Fenn indicated that if (a) the rate of CO oxidation by muscle remains proportional to the CO pressure down to the very low values met with in human blood and (b) the results on the isolated muscle at 22°C. can be extrapolated to the whole musculature of the human body at 37°C., then about 5 per cent of the CO lost from the blood in our experiments (9) might have been oxidized. Muscle *in vivo* might well be more effective than *in vitro*. We therefore thought it well worth while to apply the radioactive tracer technique to determine whether in the human body there might be any oxidation of CO to CO₂. The radioactive isotope ¹⁴C having positron and gamma ray activity and half-life of 21 minutes (10, 11, 12) was used in form³ of the labeled C*O.

In general the plan of the experiments was as follows: Several human subjects were given a relatively large labeled dose of C*O, followed by the breathing of 100 per cent oxygen, and collection in soda lime of all exhaled CO₂, and measure-

³ Radioactive carbon in form of CO will be referred to as C*O.

ment of the soda lime for any activity having a half-life of 21 minutes. The demonstration of such radioactivity in exhaled CO₂ would constitute evidence that CO is converted to CO₂ in the human body. At the same time, by placing counters over various areas on the body surface (thigh, chest, spleen, liver), the uptake and elimination of CO in these various regions were studied.

METHODS. B₂O₃ was fused to the target of the bombardment chamber of the cyclotron. On the average, bombardment with 20 mev deuterons (10 microamperes) lasted 30 minutes. The labeled carbon formed during the bombardment is in the form of CO and CO₂ and is partly trapped in the B₂O₃ target and partly in the target chamber. The latter was evacuated from the target chamber into an evacuated 300 cc. tonometer, containing 10 cc. of ordinary CO and 10 cc. of ordinary CO₂ as carriers. The tonometer had three-way stopcocks at each end.

It was essential to remove the radioactive CO₂ entirely from the gas in the tonometer before the latter was absorbed by the subject, otherwise entirely misleading results would have been subsequently obtained. The removal was done as follows: 10 cc. of 1 N NaOH were forced into the tonometer and the latter shaken violently by hand for 5 minutes. The NaOH solution was then drained off and the tonometer washed with ten successive 5 to 10 cc. portions of tap water. Each fraction was shaken thoroughly in the tonometer for some seconds before being drained off through the lower three-way tap and glass exit lead. The latter was also thoroughly washed each time through the side tube of the three-way tap so as to remove all drainage films of radioactive solution from its walls. A check as to the final purity of the gas from C*O₂ was made by adding a few cubic centimeters of ordinary CO₂ as carrier and passing the mixture slowly over soda lime. Only 0.0015 per cent of the original activity was found in the soda lime, thus indicating a very high efficiency of CO₂ removal. The slight residual activity may be due to the CO₂ absorption falling just short of the theoretical end-point or to extremely slight absorption of the C*O of the gas sample by the soda lime (or to both of these factors). Its amount was, in any case, insignificant for the purpose of the present experiments.

The activity of the remaining labeled CO was calibrated against a 1 mgm. radium standard using a gamma ray counter and a scale of eight circuit. Thereafter, it was introduced into a rubber bag into which there had previously been placed 150 cc. of ordinary CO and 2,000 cc. of O₂. The subject, after deep expiration, inhaled and exhaled in the bag three times, and then finally drew the whole contents of the bag into his lungs and held his breath for one minute. This usually resulted in an absorption of at least 95 per cent of the CO administered, and the blood CO content rose correspondingly to 2 to 3 volumes per cent. Thereafter, the subject exhaled and breathed air for seven minutes. He then was given 100 per cent oxygen and all exhaled air was passed through a glass tube (about 10 inches long and 1 inch in diameter) containing soda lime sufficient to absorb all exhaled CO₂. In the last two tests the soda lime tube was kept cool by packing in cracked ice. At 10 or 15 minute intervals a fresh soda lime tube was inserted and any radioactivity of the absorbed CO₂ was measured.

This was done with a gamma ray counter, the soda lime being poured into a glass cylinder surrounding the Geiger-Muller tube. In some experiments the total exhaled air was collected in a Douglas bag and later analyzed for CO content. Frequent venous blood samples were taken and analyzed for CO content (13, 14, 15). With the blood data, including blood volume and the Douglas bag data it was possible to calculate the amount of CO which had disappeared from the blood but which had not appeared in exhaled breath. During some of the experiments the subject was exposed to Geiger counters which pointed through a lead slit system to definite regions of the body—one counter was placed over the heart and spleen, another over the liver, and a third one over the muscles of the thigh. These made it possible to plot the curves of uptake and disappearance of labeled CO in these areas of the body.

In all activity measurements the background count was first subtracted and the resulting activity at the time of measurement, t , corrected for the decay from the starting time, t_0 , by multiplying it by $e^{0.033(t-t_0)}$, times being measured in minutes.

RESULTS OF THE EXPERIMENTS WITH C*O. Table 1 summarizes the results with respect to the possible conversion of CO to CO₂. Any CO₂ so formed would be distributed throughout the body in the form of CO₂ and HCO₃ so that only a fraction would appear in the expired air. Allowance is duly made for this factor in the table. In brief, the data show that less than 0.1 per cent of the CO lost from the blood during the first hour after CO administration is converted to CO₂. In only one instance out of 19 samples was the activity in the soda lime significantly above background (table 1, column 5). We have no explanation for this anomalous result except for a possible contamination, unnoticed at the time of its occurrence, of this particular soda lime with extraneous radioactive material. The same subject in a repeated experiment showed entirely negative results along with the other ones. The very small activities (usually less than 0.1 per cent of absorbed CO) indicating possible conversion might also be due in whole or in part to the absorption of extremely minute traces of CO by the soda lime, and the very sensitive radioactive technique detects this. These experiments however do not rule out the possibility of conversion of CO to CO₂ during longer periods of time. In two cases the urine was collected at the end of the experiment and in neither case was activity detectable.

In the interpretation of the experiments wherein the relative uptake and elimination of radioactive CO are measured, one must proceed with caution since each of the curves shown in figures 1, 2, and 3 undoubtedly contains components arising from sources other than the particular object of observation, i.e., the spleen, the liver, and the thigh. The usual type of curve observed from venous blood analyses for CO is very similar to that in figure 1. (Here the counter was placed under the posterior thigh, about 10 inches from the hip; a 1½ inch wide slit mounted crosswise to the thigh limited the solid angle of the counter which was 2½ inches from the skin.) During the first 1½ minutes there was an initial rise, probably related to the mixing of CO in the lungs. At the

end of 1½ minutes the elimination of CO started. In the thigh, (fig. 1) due to mixing and continued uptake by the blood, the activity continued to rise for

TABLE 1
*The appearance of C*O₂ in the exhaled air after breathing C*O*

NAME	DATE	C*O SAMPLE TAKEN IN THROUGH LUNGS	TIME SODA LIME EXPOSED TO EXHALED AIR	NET CTS/MIN. ABSORBED IN SODA LIME*	RATIO OF C*O ₂ FORMED TO RADIO ACTIVITY OF ORIGINAL SAMPLE**	PER CENT CO EVOLVED IN SAME PERIOD†	CO BURNT CO LOST IN SAME PERIOD
	1945	cts./min.	min.		per cent		per cent
J. H. L. (1)	3-22	1,080,000	0-15	0	0.000	28.0	0.00
M. I. G.	3-23	1,830,000	0-15	42	0.016	26.4	0.06
B. R. B. (1)	3-24	795,000	0-15	130	0.114	29.0	0.39
C. A. T. (1)	4-3	2,200,000	0-15	13	0.004	14.0	0.03
			15-30	5,600	1.820	8.5	21.40
			30-45	33	0.011	6.0	0.18
B. R. B. (2)	4-6	3,540,000	0-10	16	0.005	10.0	0.05
			10-20	34	0.010	9.0	0.11
			20-30	31	0.009	8.0	0.11
			30-40	30	0.009	7.0	0.12
			40-50	0	0.000	6.0	0.00
			50-60	0	0.000	5.0	0.00
J. H. L. (2) (soda lime cooled in ice)	4-6	2,940,000	0-15	0	0.000		0.00
			15-30	11	0.003	14.4	0.02
			30-45	26	0.006	13.0	0.05
C. A. T. (2) (soda lime cooled in ice)	4-7	3,610,000	0-15	12	0.002	14.0	0.02
			15-30	6	0.001	8.5	0.01
			30-45	0	0.000	6.0	0.00
			45-60	1	0.000		0.00
Average.....		2,230,000	in 15 min- ute period	24	0.012	14.5	0.072

* All counts/min. are corrected for decay to the starting time. Background has been subtracted.

** The C*O₂ which may form should be distributed evenly between the amount in the body and that exhaled. It was assumed that a person exhales 300 to 400 cc of CO₂ per minute, or $15 \times 300 = 4500$ cc. in 15 minutes. However, it is estimated that the total amount of CO₂ in the body may be from 27 to 33 liters. Thus, we have roughly $\frac{1}{4}$ of the CO₂ formed in a 15 minutes experiment available for measurement. The data are corrected for this dilution.

† This is the amount of CO which disappeared from the blood. It was calculated from 3 to 4 venous blood samples taken in each experiment.

a few minutes, then its drop followed one single exponential of the same order of half-time as the exponential decrease of CO in the venous blood of the arm. In

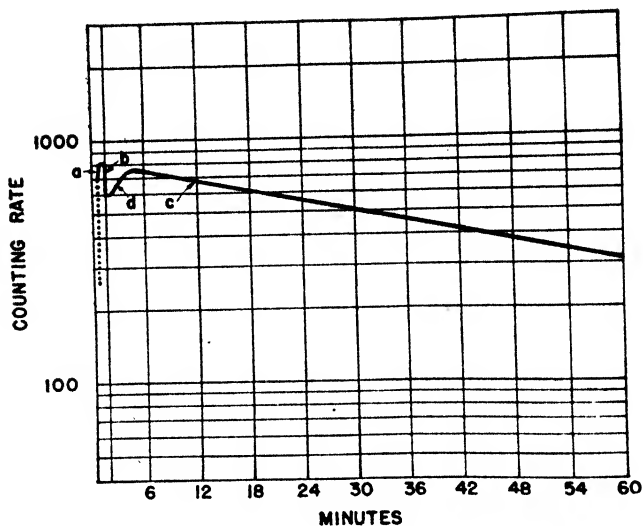


Fig. 1. Decrease of the amount of C^* in the left thigh while breathing oxygen and after $1\frac{1}{2}$ min. of inhaling C^*O . Normal subject, F. T., 22; exp. 10; 4-7-45.

- a: C^*O uptake in the lungs $1\frac{1}{2}$ min.
 b: C^*O disappears from lungs.
 c: desaturation one single exponential.
 d: continued uptake of C^*O from arterial blood.
 Half-time of desaturation: 44 min.

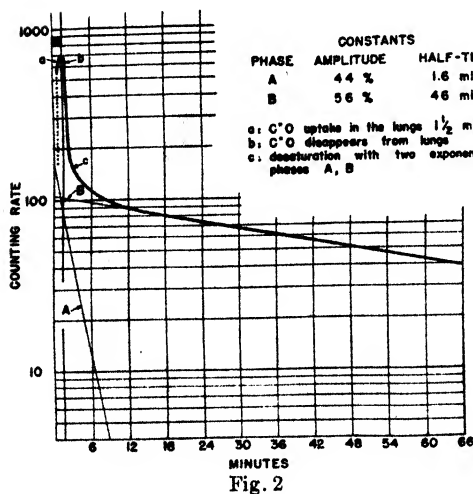


Fig. 2

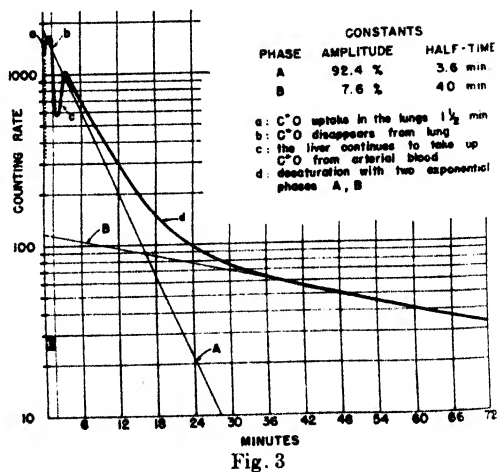


Fig. 3

Fig. 2. Decrease of the amount of C^* in the region of spleen and heart while breathing oxygen and after $1\frac{1}{2}$ min. of inhaling C^*O Normal subject, C. A. T., 27; exp. 9; 4-7-45.

Fig. 3. Decrease of the amount of C^* in the region of liver while breathing oxygen and after $1\frac{1}{2}$ min. of inhaling C^*O . Normal subject C. A. Tobias, 27; exp. 9; 4-7-45.

figure 2, the curve of the spleen-heart region, the initial high concentration due to CO in the lungs falls off with a two components curve, one fast indicating mixing (see Discussion) and a slow one similar to that in the thigh. Curves taken higher up on both sides of the chest above the spleen and the liver have similar components and may reflect chiefly the situation in heart and lung and not spleen at all. The most interesting case is presented in the liver curves (fig. 3) where the fast component has an amplitude 6 to 9 times that of the slow and where the counting rate at the beginning of the desaturation is several times higher than on the other side of the chest. It is also interesting to note that the liver region continues to take up C*O several minutes after C*O intake through the lungs has ceased. This secondary uptake might possibly be associated with the arrival of blood via the portal vein, which is thought to contribute about three-quarters of the blood supply to the liver.

TABLE 2
Elimination of C from the liver and spleen-heart regions*

NAME	LIVER					SPLEEN AND HEART					BLOOD
	Relative intensity	Fast Comp.		Slow Comp.		Relative intensity*	Fast Comp.		Slow Comp.		
		a	T _a	b	T _b		a	T _a	b	T _b	
		ampl.	half time	ampl.	half time		ampl.	half tir.	ampl.	half time	
		cts./min.*	per cent	min.	per cent		min.	cts./min.	per cent	min.	
J. H. L.....	1,700	65	4.4	35	26	900			100	24	42
B. R. B.....	10,000	91	2.6	9	91	1,650	52	2.3	48	40	70
C. A. T. (1).....	4,500	84	4.7	16	80	1,450	51	4.6	49	120	90
C. A. T. (1).....	12,500	92	3.6	8	40	1,520	44	1.6	56	46	63
Mean.....	7,180	83	3.8	17	60	1,380	37	2.5	63	57	66

* The intensities and amplitude percentages were extrapolated to the time when CO breathing was discontinued from the bag (1½ min. after the beginning of the experiment).

Table 2 presents the constants obtained in the various experiments. There seemed to be a much higher activity over the liver region at the beginning of elimination than in the spleen-heart region, although the sensitivity of the counters and the geometry with respect to the body were similar. The high initial activity and the large fast component were not expected in the liver curve, but their presence may in part explain the discrepancy between the venous arm COHb blood levels and the amount of CO eliminated in the exhaled air, the latter being found in three of the present experiments to average only 60 per cent of that lost from the blood during the first half hour after the CO administration, thus confirming the previous results of two of us (9). During the major part of the first hour the liver continued to unload CO to the general circulation. The average of the half times of disappearance of CO from the venous blood, as measured by standard techniques agree fairly closely with the average of the half lives of the slow components of the C*O curves, though in individual cases

the discrepancies mount as high as ± 40 per cent. This may be in part due to inaccuracies brought about by the movement of subjects, during the experiments, and insufficient determination of decay curves. The significance of the data in figures 1, 2, and 3 and table 2 will be further considered in the Discussion.

Measurement of elimination of CO while breathing oxygen for four hours. In view of the lack of evidence of conversion of CO in these exploratory experiments, three subjects inhaled ordinary CO, and during a four hour period thereafter exhaled CO was quantitated. Blood CO content of each subject was measured at the beginning and at the end of each experiment. Each subject took in approximately 150 cc. of CO mixed with oxygen in one case and with air in the other two. Thereafter, oxygen inhalation was continued for four hours and the exhaled air collected in Douglas bags. The volume in each bag was measured after mixing the exhaled air in it, and 1/1,000th of the volume was saved for analysis. The first bag was taken off after 10 minutes, and its CO content enabled one to calculate the amount of CO taken up by the body. All other samples were mixed, and at the end of the test the CO content of the mixed 1/1,000th fraction was measured. The results showed that in these three experiments 93, 97 and 97 per cent of all absorbed CO was recovered during this period of time in the exhaled air. The blood CO at the end of these tests was practically the same as before starting them so only a small correction was necessary on this account. These results are reported in detail elsewhere (9).

DISCUSSION. From the above results it seems clear that in mild CO poisoning only a negligible, if any, proportion of CO is burned to CO₂ in the body within the first 60 minutes of oxygen breathing. The experiments were limited to one hour owing to the relatively short half life of the radioactive carbon; oxygen was used in place of air because the elimination of CO from the blood is thereby speeded up about six times, whereas the proportion of CO recovered in the expired air is still the same, viz., 60 to 70 per cent of that currently lost from the blood (9). (It should be pointed out that the amount of CO lost from the blood in periods up to one hour is calculated from venous blood COHb values and blood volume determinations by the CO method and its accuracy therefore depends on the reliability of the latter estimations.) The absolute amount of any CO unaccounted for during the first hour is therefore six times greater when breathing oxygen than when breathing air, and the chance of finding out what happens to it should be correspondingly greater. The additional finding that almost all the absorbed CO is recovered in the expired gas if oxygen breathing is continued for four hours seems to exclude not only oxidation but also every other form of CO metabolism and seems to prove that the whole, or practically the whole, of the CO taken up by the body is reversibly combined. It will be recalled that Fenn and Cobb (5) were themselves doubtful of the applicability of their results to mammalian CO poisoning, since in the latter case the CO pressures are only of the order of one-thousandth of those they used in their muscle experiments. It is also possible that the high O₂ pressures in our experiments—both absolutely and relatively to the CO pressures—may have inhibited the catalysis,

since Negelein (8) found that for CO to be burned to CO₂ by hemins in alkaline solution the O₂ pressure must be low enough to leave the Fe only partially oxidized. Further experiments should therefore be tried at low O₂ pressures and with much larger amounts of CO in the blood. Our present results in no sense contradict those of Fenn and Cobb, but merely show that it is not feasible to make a simple extrapolation from the latter to the entirely different conditions prevailing in mammalian CO poisoning.

The more positive, though less complete and well-controlled, findings of this paper, i.e., those obtained with Geiger counters over various parts of the body seem to merit further, if perhaps more speculative, consideration. With the method of CO administration used in this paper, the alveolar CO pressure at the beginning of the breath-holding must be about 4 per cent of an atmosphere so that the COHb in the blood leaving the lungs at this stage should be of the order of 40 to 50 per cent (i.e., about 3 times the final percentage of COHb in the blood) according to calculations based on the recent data and methods of Roughton and his colleagues (16, 17). Such a rapid initial rate of CO absorption would cause the gradient of CO pressure between the alveolar air and the blood to drop rapidly, and the arterial per cent COHb must of course follow *pari passu*, being practically equal, at the end of the breath-holding, to that of the mixed venous blood entering the lung capillaries at this stage. Each organ of the body correspondingly receives blood with a high initial, but rapidly dropping, percentage of COHb and radioactivity. In organs such as the muscles, in which there are no large pools of relatively stagnant blood cells, the slow component of figures 1 to 3 should be reached as soon as the blood has circulated around the body enough times to mix the CO in it thoroughly, and the amplitude of the slow component should be proportional to the amount of blood in the organ, or more correctly, in the area of the organ which is recorded by the Geiger counter. Organs which have a rapid circulation especially if they are situated close to the heart, would be expected to show an initial high radioactivity. After a short period, however, their blood mixes with blood from the other, less well supplied regions of the body and the C*O activity then will decrease, indicating mixing. This seems to be the case in curves taken over both lungs anteriorly just below the clavicles and also over the spleen-heart region. We were hoping to detect a difference in "mixing time" between the curves obtained over the spleen-heart region, and other chest curves, since it is expected from the work of Barcroft et al. (19), on rats, that the blood stored in the sinuses of the spleen would slow down the mixing of COHb. Our failure to observe such differences may, however, have been due to insufficient accuracy in the measurements.

It is remarkable that the initial phase of the liver curve is so much more pronounced and prolonged than that of the spleen-heart curve and it is difficult indeed to see how it can be wholly explained on the same basis. In dogs and cats Barcroft et al. (18) showed that, after sudden administration of CO, the lag in COHb percentage between the liver parenchyma blood and the arterial blood only amounted to two minutes at most. These results contrast markedly

with those in the spleen (19), wherein lag periods up to 30 minutes were found, thus pointing to a much greater proportion of stagnant cells in the pulp of that organ than in the liver. No comparable figures are available for human liver and spleen though it is believed that the rôle of the spleen in man as a functional blood store is far less marked than in the dog, and it is possible that in man the liver may to some extent replace the spleen as regards blood reserve function. Even so, the ratio of the fast component to the slow component should not exceed one to one at most, unless some additional factors enter. For the highest percentage of COHb in the blood in these experiments is probably not much over 40 (see above) and, since this figure must drop rapidly during the one minute of breath-holding, the red cells of the liver can scarcely rise to higher than 30 per cent COHb by capture of CO from the cells rapidly circulating through the liver. Now the final per cent COHb reached in the mixed blood both in the liver and elsewhere averaged about 15, so that if the number of red cells in the liver that are examined by the Geiger counter remains constant throughout the experiment, the total initial activity should not have exceeded *twice* the value of the amplitude of final activity. In other words, the amplitude of the fast component would at best have been only expected to equal the amplitude of the slow component. It therefore seems that other factors must also be present if a ratio of amplitudes as high as 5 to 1 is to be explained. Two ideas suggest themselves, aside from the bare possibility that the liver handles radioactive CO in a different manner from ordinary CO: (a) The sudden arrival in the liver of blood containing a high percentage of CO might lead to a temporary engorgement of the liver with blood, (fig. 3c) followed by a contraction of the liver, expelling blood high in COHb content into other parts of the body (final steep part of curve in fig. 3). (b) The liver might contain in the Kupffer cells and elsewhere, appreciable amounts of a pigment with a far higher affinity for CO than blood hemoglobin possesses. Such a pigment would be similar to the pseudo-hemoglobin of Barkan (20), which is regarded by him as an intermediate breakdown product between blood hemoglobin and bile pigment and is calculated to have up to 10 times the affinity for CO that blood hemoglobin has. A pigment with such physico-chemical properties would be expected to take up CO very rapidly from the blood, when the COHb content of the latter is suddenly raised, and only release it slowly again to the blood as the COHb drops.

All the factors so far mentioned may well enter, in varying degrees, into the explanation of the liver curve, together with other factors which have not yet occurred to us. It is hoped to obtain further evidence in man, by simultaneous measurement of the CO content of the hepatic vein blood and other blood vessels during and after CO administration, and also by administering C¹⁸O slowly instead of in the short massive dosages used in this paper. In animals it would be desirable to carry out liver perfusion experiments and to study the affinity of liver extracts for CO directly.

Whatever may be the final explanation of the shape of the liver curve of figure 3, the matter merits further consideration in the study of acute CO poisoning and in the measurements of blood volume by the CO method.

SUMMARY

1. Several normal men were each given, via the lungs, a relatively large dose of radioactive CO, mixed with about 150 cc. of ordinary CO. Thereafter the subjects breathed oxygen for periods up to one hour, during which their expired CO₂ was absorbed in soda lime. Tests of the latter for radioactivity showed that the CO oxidized to CO₂ by the body under these conditions amounted to less than 0.1 per cent (if any) of the CO currently lost from the blood.

2. The time course of the radioactivity in three parts of the body, viz., the thigh muscles, the spleen-heart and the liver was studied by placing Geiger counters over these regions.

3. The curves for the muscle and spleen-heart areas can be reasonably interpreted in terms of the average amount of blood present in them at any given time, and the rate of blood flow. The liver curve, however, showed an unexpectedly high and prolonged initial phase, which is difficult to explain completely in such a way. The nature of the possible additional factor present in this region is discussed.

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THE RÔLE OF THE PROPRIOCEPTORS IN SHIVERING

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Shivering is not a simple spinal reflex. It may occur above, but not below, the level of a transection of the spinal cord, as shown by Sherrington and by others (13, 14, 12). There is a shivering "center" located either in the hypothalamus in cats (1), or, according to others, below the level of the red nucleus in monkeys and cats (6) and specifically in the medulla in cats (8) and in rabbits (5).

The impulses from the shivering center appear to pass down the cord via the tectospinal and rubrospinal tracts, because antero-lateral cordotomy in patients has abolished shivering below the lesion (7, 11). Bilateral shivering has been observed in patients with spastic hemiplegia (7, 11) and in dogs with unilateral and bilateral decortication (10), which indicates that the cortico-spinal tracts are not required.

The proprioceptors appear to participate in the motor mechanism of shivering. In a tabetic patient studied by Jung, Doupe, and Carmichael (7), shivering was jerk-like and irregular in the legs, in which proprioceptive function was absent. These authors conclude that afferent impulses from muscles are needed for the clonus-like movements of shivering. Similarly, Burton and Bronk (2), who studied shivering in cats by means of action potentials, state that the reflex arc may play a rôle in producing the rhythmic firing of motor units which is seen in active shivering.

While studying the mode of activation of the shivering center, Sherrington (13) observed that shivering occurred in the deafferented legs of dogs but was less than in the normal limbs. He adds the following statement: "The shivers in the deafferented limb were noted to be always synchronous with those of the normal," which implies, but does not assert, that shivering in a deafferented limb is rhythmic.

We set out to determine whether the motor mechanism of shivering resembles that of reflex clonus, as implied by Jung et al. and by Burton and Bronk. If so, cutting the proprioceptors should abolish the rhythmicity of shivering. In one group of experiments we therefore made simultaneous records of the tremor in normal and in deafferented limbs.

Moreover, if the motor mechanism of shivering does resemble that of reflex clonus, the rate of shivering should be determined by the natural rate of vibration of the moving part (limb or muscle) and not by a "pacemaker" action of the shivering center. This principle was briefly formulated for other types of tremors by Davis (3). In a second group of experiments we therefore observed whether the rate of shivering was affected by changes produced in the natural rate of vibration of a limb or muscle.

METHODS AND MATERIAL. Accurate analysis of the rate and character of shivering movements required special apparatus to record the characteristically rapid tremor. Two phonograph pickups were connected to separate channels of a Grass electroencephalograph (ink-writing oscillograph). Motion was imparted to each pickup by means of a moderately flexible steel wire substituted for the phonograph needle. The shaft of the wire was rested against a limb. In acute experiments the tip of the wire was connected to a tendon detached from its insertion. The crystal element of the pickup was mounted with its long axis at right angles to the line of motion. Movements of the limb or muscle were reproduced on the paper of the ink-writer.¹

Shivering was studied in this fashion in 24 successful experiments on 19 cats which were lightly anesthetized with 0.4 to 0.5 cc. per kilogram of body weight of veterinary nembutal (1 grain/cc.) given intraperitoneally. An equal volume of 0.1 per cent atropine sulfate was given to decrease mucus and coughing.

Shivering was induced by lowering body temperature but occurred more regularly if the cooling period was preceded by initial warming until rectal temperature reached 40°C. The front feet were then placed in water between 5 and 10°C. In most instances, shivering had started by the time the rectal temperature had fallen to 35 or 36°. If shivering continued, the rectal temperature would remain essentially constant even when the cooling procedure was continued. Animals which had not started to shiver after being cooled to 33 or 34°, rarely shivered even if cooled still further. In such instances, shivering could often be induced by repeating the entire warming and cooling process.

RESULTS. *Series I. Records of antero-posterior motions of the entire leg.* In this series, each animal was placed astride a narrow stand which permitted unrestricted movement of the hind legs. Occasionally it was also placed on its back, feet in air. The pickup wires were rested against both heels or ankles.

Group A. Normal animals (5 expts. on 3 cats). Shivering in the hind legs usually started with a twitch and developed into a fine, irregular tremor which finally became coarse and rhythmic. It frequently reverted to irregularity in one or both legs. The amplitude often waxed and waned, sometimes in relation to respiration.

The rates of contraction in the hind legs of the entire group of animals ranged from 8 to 15 per second, and were usually between 9 and 12. In the leg of a given animal the rate often varied by 2 or 3 contractions per second, sometimes by as much as 5, over a period of an hour or more.

When the shivering was truly rhythmic, the rates of contraction of the two hind legs were always equal, with the vibrations usually out of phase (fig. 1). This was also true when shivering was recorded in the feet alone, by immo-

¹ For testing the accuracy of the recorders, the tips of the pickup wires were mechanically connected to the moving part of a separate ink-writing unit. This unit was then driven by an oscillator. A sine wave was faithfully reproduced at frequencies between 3 and 45 cycles per second. A satisfactorily flat "frequency response" was obtained by connecting 5 megohm resistors in series with both leads of each pickup, and by appropriate use of the filters built into the amplifiers for the purpose of attenuating high frequencies. Shielded cable was used throughout.

bilizing the ankles with needle-pointed clamps. However, when a weight was attached to one foot, thereby decreasing its natural rate of vibration, or "resonant frequency," the rate in the two feet became unequal. Figure 2 shows bilateral rhythmic shivering at a rate of 10.4 per second in the weighted foot, 12.4 in the normal.

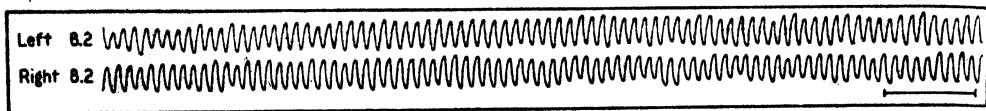


Fig. 1. Simultaneous records of shivering movements of both entire hind legs, normal animal. Numbers indicate contractions per second, based on 10 second counts. Time calibration: 1 second, in this and following figures.

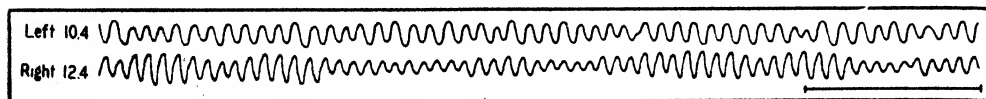


Fig. 2. Shivering movements of both feet with weight attached to left; normal animal, ankles immobilized.

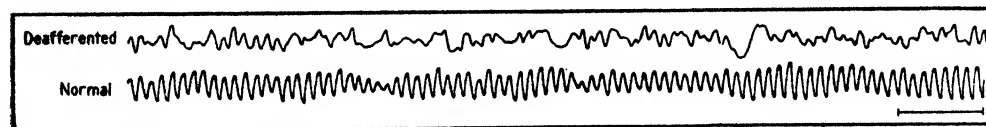


Fig. 3. Shivering movements of both entire hind legs, showing lack of rhythmicity in the deafferented side.

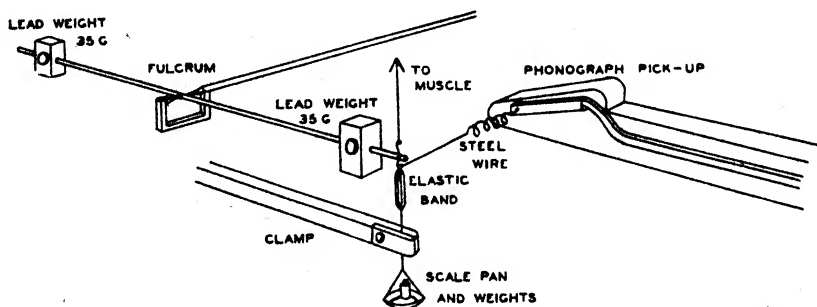


Fig. 4. "Mechanical resonator" for producing changes in the resonant frequency of a moving muscle, with phonograph pickup arranged for recording the tremor.

Group B. Animals with one deafferented hind limb (8 expts. on 6 animals). Laminectomy and unilateral section of all dorsal roots below L_3 were performed aseptically on 4 animals. Anesthesia extended over the deafferented limb, movements of which were ataxic when the animal walked. Experiments were performed approximately one month after operation. In 2 acute experiments dorsal root section was performed on the day of the experiment.

Results. Totally irregular movements characterized the shivering of deafferented limbs, in contrast to the rhythmic movements seen in normal limbs. Large, flail-like motions were superimposed upon an irregular, fine tremor. This type of shivering closely resembles that described in the legs of the tabetic patient studied by Jung et al. (7).

Figure 3 shows shivering recorded simultaneously in a normal and in a deafferented limb. In the many hours of recording only a few seconds of rhythmic motion have been observed in a deafferented limb. In most cases, these ap-

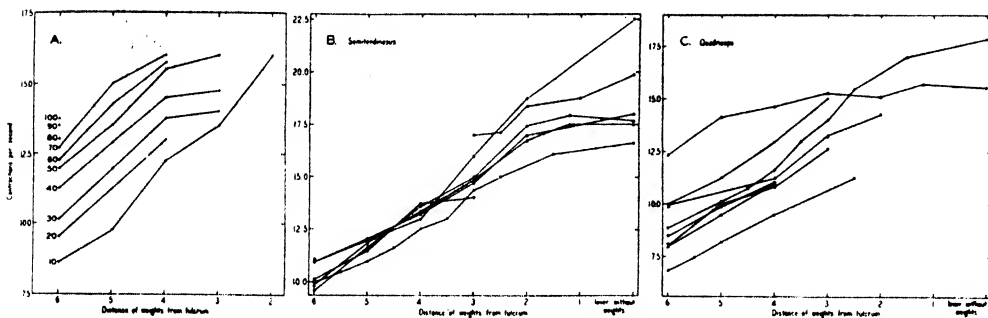


Fig. 5A. Rates of contraction in a single semitendinosus muscle plotted against distances (in cm.) of weights from fulcrum. Each curve was obtained at constant tension, indicated in grams by the numbers to the left.

Figs. 5B and 5C. Curves obtained in different animals at essentially the same tension (20 or 30 grams in each).

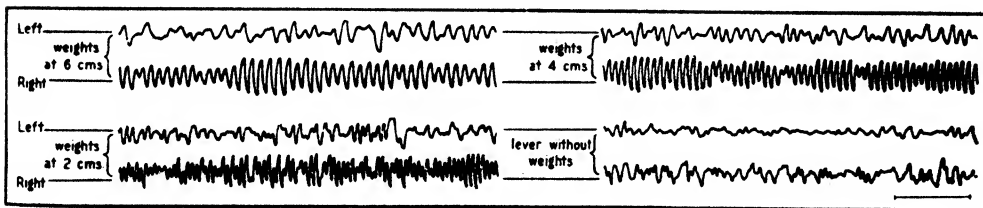


Fig. 6. Shivering movements in deafferented (left) and normal (right) semitendinosus muscles with resonators on both. Tension 10 grams.

peared to be vibrations transmitted from other parts of the body, usually from the opposite, normal leg.

Group C. Hemidecerebellate cat. Dworkin (5) found normal shivering in a rabbit after removal of the cerebellum. On the other hand, Uprus et al. (11) and Jung et al. (7) observed "choreiform movements" in place of normal shivering in patients with cerebellar disease. To obtain additional information on this point, we performed an experiment on a cat with chronic removal of one half of the cerebellum.² Although the signs of the cerebellar deficiency were in every

² This and one of the animals with a deafferented limb were kindly loaned by Dr. R. S. Morison.

way typical in the unanesthetized animal, the shivering recorded was equal in both legs, rhythmic and apparently entirely normal.

Series II. Effects of changing the "resonant frequency" of individual muscles or muscle groups. The tendons of the semitendinosus muscles and of the quadriceps were freed from surrounding tissues and were liberally coated with light mineral oil, U.S.P., to prevent drying. The animal was placed upright astride the stand and the femora were immobilized by means of drills. The tendons of any two muscles, usually the left and right members of a pair, were attached to the two pickup wires.

The tendon was attached to one end of a light lever, 15 cm. long (fig. 4). A rubber band under tension was hooked to the under side of the same end of the lever. Contractions of the muscle produced teetering motions of the lever. The lever and muscle, together with the rubber band, constituted a resonating system, which, like the balance wheel of a watch, possessed its own natural rate of vibration, or "resonant frequency."

Two separate methods were available for altering the resonant frequency of the system. The first method consisted of adjusting the position of a pair of weights mounted on the lever at equal distances from the fulcrum. Moving the weights closer together increased the resonant frequency of the system, i.e., the lever tended to vibrate more rapidly. The second method consisted of changing the tension of the rubber band by adding or subtracting weights from a scale pan hung from its lower end. (The pan was clamped to prevent bouncing when records were taken.) Increasing the tension increased the resonant frequency of the system.

Myotatic reflexes could be obtained by tapping the lever; but all attempts to elicit a reflex clonus failed.

Results: Group A. Normal animals. (4 acute expts.) With the resonator attached the movements of single muscles were clearly rhythmic except in the mildest shivering. Figure 5A consists of a family of curves obtained from a single muscle and shows that, with tension kept constant, the rate of shivering movements could be greatly increased (sometimes by as much as 100 per cent) by decreasing the distance of the weights from the fulcrum of the lever. Conversely, with a constant setting of these weights, increasing the tension also greatly increased the frequency of the contractions, sometimes by as much as 100 per cent. Figures 5B and 5C show curves, each determined on a normally innervated muscle of a given animal at essentially the same tension (20 or 30 grams).

The highest rate of rhythmic shivering which we have recorded was 28 contractions per second (the lever was disconnected) and the lowest rate was 6 per second.

Although the rate of shivering in a given entire leg had been found to vary during the course of a prolonged period of recording, it is noteworthy that the rate in an individual muscle was essentially constant with a given setting of the resonator. This was true regardless of how hard the animal was shivering, or whether an hour or more had elapsed between taking two records. The maxi-

imum deviation was of the order of 5 to 15 per cent of the average rate. Part of this deviation may have been caused by factors arising within the animal, but a certain part was undoubtedly caused by inability to reproduce a given setting of the resonator with complete accuracy.

We had observed that the two hind legs of a normal animal tended to shiver at identical rates, but we found no such tendency in two individual muscles. For example, in each of two animals, the rates in corresponding left and right muscles were slightly unequal with apparently the same tension in both. (The levers were disconnected.) In 2 other animals³ with complete resonators on both sides, slightly unequal rates were observed with apparently identical settings, for example 17.4 and 18.1 in left and right quadriceps respectively. The complete independence of one side from the other was still further illustrated by the widely differing rates, for example 13.5 and 21.5 in left and right quadriceps respectively, which appeared when widely differing settings were used. Beats indicating interference between sides did not occur.

Group B. Animals with one deafferented hind limb, resonators attached (4 acute expts.). With normal animals we had observed that shivering was more likely to be rhythmic in a muscle attached to a resonator than in an entire leg. We therefore used resonators on deafferented muscles to determine whether rhythmic shivering might possibly occur. Two resonators were usually employed in a given experiment, one attached to the deafferented muscle, the other to the normal muscle. Simultaneous records were made, keeping the various settings equal in both resonators. Typical results are shown in figure 6.⁴ No rhythmic shivering lasting more than a few contractions ever appeared in a deafferented muscle.

Group C. Animals with section of one dorsal column of the cord (2 acute expts.). Unilateral section of the columns of Goll and Burdach at a level of C₂ was performed aseptically. An effort was made to include somewhat more tissue than necessary. One animal showed slight ataxia in the front feet, the other no demonstrable abnormality in gait. Experiments were performed approximately one month later. As stated under group A, series II, the lesion had no demonstrable effect on shivering.

DISCUSSION. The above experiments appear to confirm the conclusions of Jung et al. (7) and of Burton and Bronk (2) who stated that rhythmic shivering will not occur without afferent impulses from muscles. A pacemaker action of the shivering center appears unlikely, because in a given animal two muscles could be made to shiver at slightly or widely differing rates at the same moment.

* Section of one dorsal column in the neck had been performed on these animals, hence they were not strictly normal. However, shivering appeared to be normal on both sides. Data obtained are therefore included because it was assumed that equality of rhythm in opposite sides would probably be produced by connections within the cord and not by long proprioceptive pathways.

⁴ In figure 6, the record of the normal (right) side is a portion of that used to construct the 10 gram tension curve shown in figure 5A. With the weights removed from the lever, the normal muscle is here unable to sustain a regular rhythm. This phenomenon frequently appeared at the high and low frequencies of the resonator.

It is more reasonable to assume that the shivering "center" in the brain sends out an irregular stream of facilitating impulses which produce irregular firing of anterior horn cells until the proprioceptors are activated sufficiently to synchronize this firing.

Such a process resembles a reflex clonus in some respects. The rhythmicity and rate are determined peripherally. Because the rate tends to follow the natural rate of vibration of the resonator, but does not vary with changes in the intensity of shivering, the rate is probably close to the resonant frequency of the moving part.

The variation in the rate of shivering, which occurred from time to time in an entire leg, may appear to contradict this statement. However, spontaneously occurring changes in the degrees of flexion of the joints, accompanied by proportional changes in the resting tensions of the various muscles, would necessarily alter the resonant frequency of a leg and thereby its rate of shivering. When the resonant frequency was kept relatively constant, (i.e., by using a resonator at a given setting of weights and tension) these variations in rate were largely eliminated.

In our experiments, the left and right hind limbs of normal animals were found to shiver at the same rate. Others have reported a similar identity of rhythm in two or more limbs of human subjects during periods of intense shivering (4, 9). We did not, on the other hand, observe the phenomenon in two individual muscles. This suggests that the mechanism responsible for equal rates of shivering in different parts of the body requires the activity of relatively large numbers of proprioceptors, which would be provided by the motions of an entire leg but not by those of an individual muscle.

I wish to thank Drs. Hallowell Davis and George H. Acheson for their many helpful suggestions.

SUMMARY

1. Shivering movements in cats were recorded by means of two phonograph pickups connected to a two-channel ink-writing oscillograph.
2. Simultaneous records of the tremor were made in normal and in deafferented hind limbs. The movements of the limbs during shivering were rhythmic on the normal side, always irregular on the deafferented side.
3. A hemidecerebellate animal and two animals with severance of one dorsal column shivered normally on both sides.
4. The rate of shivering in a normal muscle could be changed by 100 per cent or more by varying the mechanical resonance of the moving muscle. This was accomplished by adjusting the position of a pair of weights mounted on a lever attached to the tendon, or by varying the tension of a rubber band against which the tendon pulled. Deafferented muscles did not shiver rhythmically even with this arrangement.
5. It is concluded that the rhythmicity and rate of shivering are determined peripherally by a mechanism which involves the proprioceptors and resembles

that of reflex clonus. The rate of shivering in the normal animal is probably close to the resonant frequency of the moving part. A pacemaker action of the shivering center appears unlikely.

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ON THE THROMBOPENIA OF ANAPHYLACTIC AND PEPTONE SHOCK¹

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Three characteristic findings in both anaphylactic and peptone shock are: a sudden release of histamine, an outpouring of heparin into the blood stream, and a precipitous decrease of the circulating platelets. The intensity of the three effects is apparently not related, contrary to the hypothesis expressed by the senior author in his monograph (1) that histamine is a factor in the causation of the marked thrombopenia of anaphylactic shock. The sudden and striking decrease of circulating platelets, although repeatedly noted by various investigators has received little attention. In the present work an attempt has been made to learn more about the nature of this phenomenon and especially to determine whether the decrease of platelets in peptone and anaphylactic shock can be prevented by heparin.³

METHODS. *Peptone shock.* The dog was given 300 mgm. of Witte peptone per kilo of body weight intravenously. The peptone was dissolved in 5 parts of 0.85 per cent saline solution, and clarified by high centrifugation.

Anaphylactic shock in rabbits. The animals were sensitized by the injection of 0.5 cc. horse serum intravenously every two or three days until 6 doses were given. Shock was induced by the intravenous injection of 3 cc. of horse serum 10 to 14 days after the last sensitizing dose.

A few guinea pigs were sensitized by the injection of 1.5 cc. of egg albumin intraperitoneally. Shock was induced by giving 1.5 cc. egg albumin intravenously approximately three weeks later.

Platelet count. One cubic centimeter of blood was drawn into a 2 cc. syringe coated with a thin film of mineral oil and containing 0.5 cc. of 3.8 per cent

¹ A preliminary report of this work appeared in Fed. Proc. 3: 62, March 1944.

² Doctor Ota is now serving with the armed service, and Dr. Baronofsky is in the Department of Surgery, University of Minnesota Medical School.

³ The heparin employed in this study was Roche Organon Liquaemin which contains 10 mgm. per cc. It was kindly supplied by Mr. Edward A. Wickham of Roche Organon Inc., Nutley, New Jersey.

sodium citrate solution. The blood was quickly transferred to a small test tube coated with collodion and immersed in ice. The blood was diluted 1 to 200 with 3.8 per cent sodium citrate and the count made according to the Rees and Ecker procedure.

Splenectomy and peptone shock. In order to determine whether the spleen was a factor in the production of thrombopenia in peptone shock, several dogs were splenectomized and five days later injected with peptone. The results are given in table 1. Dogs 1 and 2 showed little systemic reaction whereas 3

TABLE 1
The effect of peptone shock on the platelets in splenectomized dogs

MINUTES AFTER INJECTION OF PEPTONE	PLATELET COUNT				
	Dog 1	Dog 2	Dog 3	Dog 4	Dog 5*
0	700,000	528,000	405,000	442,000	538,000
5	114,000	159,000	33,000	51,000	66,000
30	642,000	584,000	261,000	162,000	500,000

* Normal dog as control.

TABLE 2
The effect of heparin in peptone shock

TIME	DOG 6*		DOG 7† PLATELET COUNT
	Platelet count	Prothrombin time	
		sec.	
Before injection of heparin.....	390,000	6	405,000
30 min. after injection of heparin.....	315,000	18	400,000
1 min. after injection of peptone.....	282,000	13	63,000
5 min. after injection of peptone.....	90,000	23	96,000
30 min. after injection of peptone.....	312,000	11	500,000

* Dog 6 weighed 11½ kgm. He received 6 cc. of heparin (60 mgm. or 6000 units) which is 520 units per kilo of body weight. The animal had a severe reaction—marked peripheral hyperemia, vomiting, involuntary defecation, and tremors.

† Dog 7 weighed 6 kgm. She was given 5 cc. of heparin (50 mgm. or 5000 units) which is 833 units per kilo of body weight. Only a mild systemic reaction occurred.

and 4 were affected severely, particularly dog 4 in which vomiting, involuntary defecation and marked prostration occurred. An outpouring of heparin into the blood as measured by the writer's thrombin titration method (1) was found in all the animals, but it was most pronounced in dog 4.

The effect of the intravenous injection of peptone into splenectomized dogs is indistinguishable from that in normal dogs. It can therefore be concluded that the spleen is not a factor in the reduction of platelets in this type of shock.

Heparin and peptone shock. To test whether a large dose of heparin given intravenously can prevent the fall in platelets caused by peptone shock, a series of well-fed dogs were first injected with heparin and then subjected to peptone

shock. Two representative experiments were selected for presentation in table 2.

Obviously heparin does not protect against the thrombopenia of peptone shock. Since the prothrombin time can serve as a measure of the heparin in plasma provided the prothrombin remains constant, it can be concluded that in dog 6 the injected heparin also failed to prevent further outpouring of heparin into the blood stream following the injection of peptone. Heparin obviously does not influence the effects or course of peptone shock in the dog.

TABLE 3

The influence of hypoprothrombinemia (due to dicumarol) on peptone shock

MINUTES AFTER INJECTION OF PEPTONE	DOG 8 (WEIGHT 8 KGM.)	
	Platelet count	Remarks
0	270,000	The dog was given 80 mgm. of dicumarol orally for 4 days. On the seventh day, the clotting time of recalcified plasma was 270 seconds (normal 90 seconds); clotting time (Lee White) 12½ minutes (normal 3½ to 6 minutes); prothrombin time 85 seconds (normal 6 seconds).
10	12,000*	
30	101,000	

* The animal had a severe reaction. He passed blood by rectum. The dicumarol was kindly supplied by Eli Lilly and Company.

TABLE 4

The effect of heparin on the thrombopenia of anaphylactic shock in rabbits

ANIMAL.....	1	2	3	4	5	6	7	8*
Weight in kgm.	2.8	2.7	2.7	2.5	3.4	2.7	3.0	3.1
Heparin.....				3 cc.	2 cc.	2 cc.	2 cc.	
Minutes after injection of shocking dose	Platelet count							
0	456,000	504,000	522,000	525,000	417,000	543,000	441,000	387,000
5	150,000	72,000	60,000	51,000	123,000	75,000	39,000	381,000
Reaction	Severe	Weak	Moderate	None	Weak	None	Moderate	None

* Nonsensitized rabbit as control.

Dicumarol hypoprothrombinemia and peptone shock. The object of this experiment was to ascertain whether a drastic reduction of the coagulability of the blood by means of feeding dicumarol to a dog made the animal less susceptible to peptone shock. In table 3 the results of such an experiment are recorded. It can be seen that neither the severity of the shock nor the thrombopenia was influenced by the hypoprothrombinemia and the resulting hypo-coagulability of the blood.

Heparin and anaphylactic shock. In rabbits sensitized to horse serum the injection of the shocking dose of the antigen consistently caused a drop in the

circulating platelets. As seen in table 4, the injection of heparin did not influence the thrombopenia. Interestingly the fall in platelets was marked even when the systemic reaction was mild or almost absent.

Histamine and the platelet count. When histamine was injected intravenously the platelets did not decrease, in fact there was a tendency to increase slightly which is probably explainable by the transient hemo-concentration which is likely to occur (table 6).

TABLE 5

The effect of heparin on the platelet count in anaphylactic shock produced in guinea pigs*

MINUTES AFTER INJECTION OF SHOCKING DOSE	PLATELET COUNT	
	Guinea pig 1	Guinea pig 2
0	300,000	400,000
5	72,000	126,000

* Each animal was given 0.5 cc. of heparin intravenously 30 minutes before the shocking dose of egg albumin was given. Both animals died in shock.

TABLE 6

The influence of histamine on the platelet count

MINUTES AFTER INJECTION OF HISTAMINE*	PLATELET COUNT			
	Dog 1	Dog 2	Dog 3	Dog 4
0	377,700	201,250	485,000	475,000
5	403,000	293,730	515,000	345,000

* One milligram of histamine per kilo of body weight was injected intravenously.

DISCUSSION. In both peptone and anaphylactic shock, a precipitous decrease in the circulating platelets is a characteristic finding. If the blood is collected just before the drop occurs, the platelets are found adhering to each other forming clumps of varying sizes. This was also observed by Eagle, Johnston and Ravdin (4) and others. It is very probable that the decrease in platelets is due to this agglutination with subsequent removal of the clumps by the capillary bed. Whether the rather prompt restoration of the platelet count is due to a replenishment of new ones from the bone marrow or a dispersion of the agglutinated cells is not known.

The actual agent or factor responsible for the agglutination and subsequent decrease of the platelets has not been identified. It is fairly certain, however, that histamine is not responsible, for the platelets remain normal in shock induced by the injection of histamine as shown by Kinsell *et al.* (3) and confirmed in this study. Furthermore, there appears to be no correlation between the degree of thrombopenia and the severity of the reaction, which is held by many to be due to the release of histamine. In many of the rabbits sensitized to horse serum, the shocking dose caused no detectable systemic reaction but did

produce a marked thrombopenia. It should be mentioned, however, that Kinsell and his associates found that in anaphylactic shock in the monkey, there did exist a relationship between the degree of thrombopenia and the severity of the shock, but it is probable that this was coincidental.

It is now known with certainty that the delay in coagulation observed in peptone and anaphylactic shock is due to the outpouring of heparin into the blood stream. The mechanism of this action is not known, nor has it been determined whether the liberated heparin serves a useful purpose. It seemed possible that heparin might serve to counteract the agglutination of platelets in shock. *In vitro* the platelets are kept discrete by heparin provided 0.25 mgm. per cc. of blood is used (4). Solandt and Best (5) have further demonstrated that the agglutination of platelets by a foreign body or surface (glass cell technique) was prevented *in vivo* when the dog was given 300 units of heparin (3 mgm.) per kilo of body weight intravenously.

In the present investigation it was found that doses of heparin considerably larger than those effective in preventing platelet agglutination on the transverse scratch of the glass cell of Best and Solandt did not prevent the drop in platelets and presumably the agglutination of platelets which precedes the thrombopenia in either peptone or anaphylactic shock. Likewise in the dog with a markedly delayed coagulation time due to hypoprothrombinemia from dicumarol, the platelets decreased drastically when a shocking dose of peptone was injected. On the basis of these findings it would appear that the agglutination of platelets in shock differs from the formation of white thrombi caused by a foreign body or surface. The latter can be prevented by anticoagulants whereas the clumping of platelets in shock is apparently not inhibited by these agents. It appears, therefore, that there may be two types of platelet-agglutination, one caused by a foreign surface, the other by an agent produced by the body in response to an immunological stimulus.

The thrombopenia of shock is of clinical interest because of the possibility of a relationship to thrombocytopenic purpura. It is known that allergy and drug sensitivity are causes of this hemorrhagic condition and it seems reasonable to suppose that the same fundamental mechanism is operative in the idiopathic type known as Werlhof's disease. The characteristic features of both the primary and the secondary types of purpura is the marked diminution of circulating platelets. Since the bone marrow is normal or hyperplastic (except in the aplastic type) the depletion of platelets is very likely not due to a lack of production but rather to a rapid removal similar to the condition in experimental shock. There is much evidence that the bleeding tendency, i.e., the hyperpermeability of the capillaries is not directly dependent on the decrease in platelets but rather that both the thrombopenia and the vascular dysfunction may perhaps be caused by the same agent. In this it likewise bears a similarity to peptone and anaphylactic shock in which conditions the thrombopenia, heparinemia and histamine release appear to be relatively independent of each other but probably are produced by one and the same basic agent.

SUMMARY

1. In both peptone and anaphylactic shock, a sudden and severe decrease in the circulating platelets occurs. The appearance of platelet clumps prior to their disappearance suggests that agglutination precedes their removal.

2. Splenectomy has no effect on the thrombopenia of peptone shock in dogs.

3. The intravenous injection of large doses of heparin does not prevent the decrease of platelets in peptone or anaphylactic shock.

4. The intravenous injection of histamine does not cause a decrease of the platelets in the blood.

5. The possible bearing of the results obtained in this study to thrombocytopenic purpura is discussed.

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THE RÔLE OF EXERCISE IN ALTITUDE PAIN

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Exposure of humans to barometric pressures of less than one-half atmosphere while maintaining normal alveolar oxygen tension frequently results in the occurrence of intense pain (aviators' "bends"), usually localized in or near the joints (2). This pain is considered to be a "normal" response to an unusual environment (4), increasing in frequency and severity in proportion to the reduction in pressure (3). Under standard conditions of exposure, some individuals repeatedly experience pain while others are relatively immune (7). The pain is thought to be the result of expanded tissue gases in the form of extravascular bubbles (9) or embolic ischemia of osseous tissues resulting from intravascular bubbles (1). The occurrence of the pain can be partially prevented by removal of gaseous nitrogen through pre-breathing of pure oxygen for several hours (6).

It has recently been shown that altering the frequency of exercise bouts that occur during exposure to reduced pressure influences the incidence and severity of pain in humans (3). Intravascular bubble formation has been observed in animals at a simulated altitude of 20,000 ft. (350 mm.) when violent muscular activity occurred, while no bubbles were observed at 50,000 ft. (88 mm.) if muscular activity was slight (10). The rôle of high local concentrations of CO₂ resulting from exercise has been stressed by the Stanford group of investigators (5, 10); the Princeton investigators on the other hand emphasize mechanical tension as the most important factor (8). Both groups have largely based their conclusions on visual demonstration of intravascular bubbles in animals—frogs and rats in the Stanford experiments, and cats in the Princeton work. In the experiments to be described, the problem will be approached by systematically varying the nature and severity of exercise, observing the effect of such variation on "bends" pain experienced by human subjects.

METHOD. The subjects, all healthy young men, were exposed to a barometric pressure of 155 mm. (equivalent to 38,000 ft. altitude) in a decompression chamber. Pure oxygen, administered by mask, was supplied except during the first 10,000 ft. of ascent to the simulated altitude. The ascent to 38,000 ft. required 12 minutes; the men remained at that altitude for 90 minutes unless forced to return to ground level pressure sooner because of incapacitating symptoms. A standard exercise was performed as soon as the decompression chamber

¹ The work described in this report was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the University of California. J. H. Lawrence, Director, and other members of the unit co-operated generously in conducting the experiments.

reached 38,000 ft., and was repeated at intervals of $2\frac{1}{2}$ minutes (controlled by an automatic timer). No other activity was permitted; the subjects remained seated throughout the experiment. The presence and severity of pain or other symptoms was recorded at intervals of $2\frac{1}{2}$ minutes. All of the men had previously been indoctrinated in the procedure, and had done one standard exercise immediately before the ascent. Additional details concerning the procedure will be found in other reports (2, 3, 7).

All six of the exercises were done with a metal bar 1.5 inches in diameter and 16 inches long that normally weighed 8 lbs.; rectangular weights cut from half-inch sheet lead were attached to the ends to increase the total weight to 16 or 36 lbs. as desired. In the first four types of exercise the bar was manipulated in cadence with a metronome set at 80 beats per minute, as follows:

- (a) The bar was held by both hands, palms up, in a horizontal position, 2 inches above the knees.
- (b) Raised by elbow flexion to a position 2 inches in front of the eyes.
- (c) Thrust upward nearly vertically, until the elbows were completely extended.
- (d) Returned to the eye position.
- (e) Lowered to the knee position (but not permitted to touch the knees).

Two of the exercises involved 8 of the above cycles in a 24 second period; two were done in "slow motion" so that only 4 cycles occurred in 24 seconds. The fifth exercise involved raising the 36 lb. bar a few inches and holding it quietly for 24 seconds; the supplementary end weights on this bar, resting on forward extensions attached to the sides of the chair, held it in a convenient position a few inches above the knees when it was not being manipulated. In the sixth exercise, the 36 lb. bar was raised 2 inches by elbow flexion and returned to rest on the chair extensions; sixteen such cycles were done in 24 seconds. This last exercise was done in a "snappy" manner, whereas in the fifth exercise the bar was raised rather gradually with sudden strain at a minimum. Care was taken to insure that all exercises were always done in a well-standardized manner. A quantitative comparison of the six exercises is made in table 1.

Since the first exposure to low barometric pressure is known to result in greater incidence and severity of pain than subsequent ones, the exposures have been balanced in such a way that first exposure occurred for about half the men doing each exercise; the other half were usually being subjected to their second exposure, although it was occasionally their third and in a very few cases their fourth or fifth. Aside from this balancing, successive exposures of each man were distributed among different exercises in a random manner.

EXPERIMENTAL RESULTS. Sixty-three per cent of the total exposures resulted in pain in the hand, wrist, elbow or shoulder, whereas only 16 per cent resulted in pain in the foot, ankle, knee or hip. Pain of moderate or greater severity in the arms occurred to the extent of 42 per cent; in the legs, 7 per cent. Incapacitating pain in the arms occurred in 22 per cent of the exposures, and in the legs, only 2.5 per cent. In other words, 80 per cent of all pain, 86 per cent of moderate or severe pain, and 90 per cent of incapacitating pain occurred in the arms.

This finding may be compared with the results of an earlier experiment involving only leg exercises; in the latter case 82 per cent of the joint pains reported were localized in the legs (2). Thus the evidence is unequivocal in showing that

TABLE 1
Quantitative description of arm exercises and incidence of arm symptoms

EXERCISE	WT.	CYCLES	TOTAL DISTANCE	FT. LBS.	WT. X JERKS	MILD	MODERATE	INCAPACI- TATING	NO. MEN EXPOSED
	lbs.		ft.			%	%	%	
I	16	8	23.8	380	256	72.4	55.3	40.8	77
II	16	4	12.7	204	128	70.5	54.1	27.9	62
III	8	8	23.8	190	128	74.1	44.9	22.4	61
IV	8	4	12.7	101	64	53.1	34.4	10.9	65
V	36	1	0.2	7	36	59.8	29.9	13.4	69
VI	36	16	2.7	97	576	43.6	27.3	14.5	56

Note: Effective weight of the arms has been neglected. Work of picking up the bar has been included in total distance and foot-pounds. Angular movement of joints is related to total distance. In the case of exercise V, total distance and foot-pounds are probably considerably greater than the tabled values because the heavy bar could not be held without some movement. Measurements on two individuals showed an oxygen cost of 230 cc. for V and 240 cc. for IV.

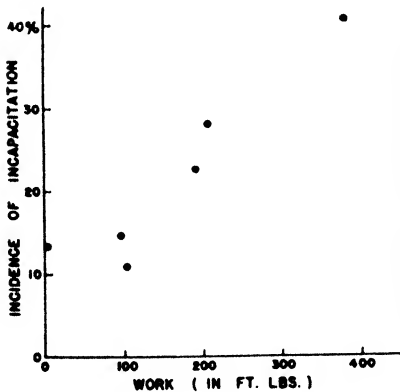


Fig. 1

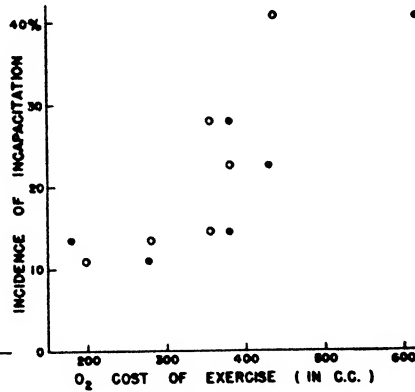


Fig. 2

Fig. 1. Incidence of incapacitating pain in relation to calculated work.

Fig. 2. Incidence of incapacitating pain in relation to oxygen cost of the work measured on 2 men.

altitude pain may occur in unexercised limbs, but it is far more frequent and more severe in limbs that are exercised intentionally.

Symptom producing effects of the different exercises are shown in table 1. Analysis of this table reveals a high association between incidence of incapacitating symptoms and foot pounds of work (fig. 1). A few direct measurements of

the oxygen cost of the exercises are available.² The results (fig. 2) are similar to those based on calculated work. Total symptom incidence is also associated fairly well with work in foot pounds, although the incidence for exercise I is not as high as would be expected under the assumption of a linear relation between incidence and work, and is too high for exercise V because of the inclusion of a number of cases of low grade pain that are probably not true altitude pain. The latter exercise was observed to produce mild pain of ischemic origin at sea level because of the sustained isometric muscular contraction.

Symptom onset time, also, is related to work done. The relation is clearest with early appearing symptoms; when late appearing symptoms (which are usually mild) are included, there is no differentiation between exercises I, II and III, but these three are differentiated from IV, V and VI. In figure 3 cumulative

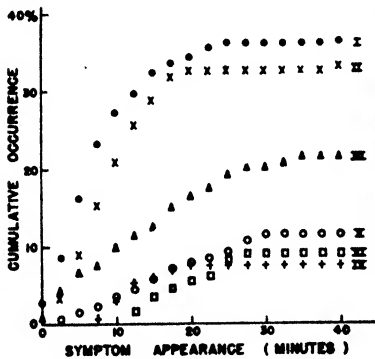


Fig. 3

Fig. 3. Time of first appearance of pain that later became at least moderately severe.

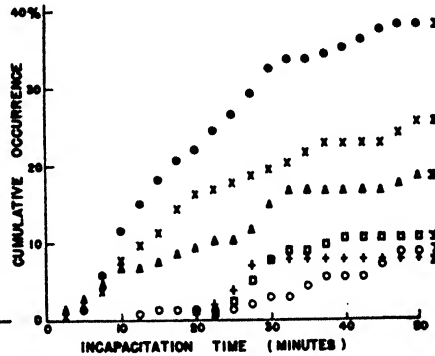


Fig. 4

Fig. 4. Time of incapacitation.

frequency curves based on onset times of moderately severe symptoms are shown to be related to work done in the different exercises; the association is somewhat clearer in the case of incapacitation time, as shown in figure 4.

DISCUSSION. The data are best accounted for by a theory that assumes that exercise increases symptom incidence and severity and hastens the onset of symptoms through a mechanism dependent upon the rate of work or total work done.³ The animal experiments of the Stanford investigators (5) suggest that CO_2 produced by the working muscles is the responsible agent.

H. B. Jones of this laboratory has calculated that the local concentration of CO_2 in the muscles resulting from the exercises used in the present experiment is adequate to account for the observed results.

Strain or mechanical tension, as such, does not appear to be an important

² The writer is indebted to W. E. Berg for the oxygen consumption measurements.

³ Paradoxically, exercise also hastens the rate at which symptoms disappear after reaching their maximum (6). Presumably this is due to the nitrogen-flushing function of the circulation which is augmented by exercise.

factor in the present experiment. Exercise VI involved severe, frequent, intermittent strain and tension to a far greater degree than did any other exercise, yet it is one of the mildest insofar as symptom production is concerned. Continuous severe strain, as exemplified by exercise V, appears to have no unique effect on symptoms. The product of weight and number of intermittent movements is unrelated to symptom occurrence; exercise VI is very different from IV and V in this respect, but about equal in symptom-producing effects. Turbulence (5) must have been minimal in V and maximal in VI, yet symptom occurrence is almost identical with these two exercises. Angular movement of joints cannot be important in itself, since exercises II and IV, equated in this respect, vary greatly insofar as symptoms are concerned. Exercises IV, V and VI involve similar amounts of work; they are similar also in terms of altitude pain. Doubling the work of exercise IV by increasing the weight of the bar (II) or the number of movements (III) correspondingly affects the pain; a further doubling of the work (exercise I) is reflected in altered occurrence of the more severe types of pain. Only work in foot pounds, or some factor directly related to it, is systematically related to the occurrence, severity, and time aspects of pain symptoms observed in this study.

The evidence presented above is supported by another experimental study, in which several combinations of arm and leg exercises were used (7). Equal incidence of pain in the upper and lower extremities occurred with thirty cycles of the exercise III movements and five cycles of step-ups on a 9 inch stool. These two exercises are almost identical with respect to total work, but obviously differ considerably in other respects.

SUMMARY AND CONCLUSIONS

Young men were exposed a total of 390 times to a reduced barometric pressure equivalent to an altitude of 38,000 ft. in order to determine the aspects of muscular exercise that effect the occurrence of altitude joint pain (aviators' bends). Pure oxygen was supplied so that alveolar oxygen was maintained at normal ground level tension. While at the simulated altitude, the men performed one or another of six arm exercises designed to separate out factors such as continuous and intermittent strain, total work, and angular joint movement. The results lead to the following conclusions:

1. Pain occurs in unexercised limbs, but is much more common and more severe in exercised limbs.

2. Onset time, incidence and severity of pain are systematically related to total work (in foot-pounds). There is an indication that the occurrence of mild pain is differentially affected by small amounts of work, but not further increased at the highest work level studied.

3. Muscle strain, mechanical tension and amount of joint movement do not determine the occurrence or severity of pain. This is true for both continuous and intermittent tension.

4. The results support a theory that increased local CO_2 production is principally responsible for the effects of exercise on the occurrence and severity of altitude pain.

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THE ELIMINATION OF ADMINISTERED COBALT IN PANCREATIC JUICE AND BILE OF THE DOG, AS MEASURED WITH ITS RADIOACTIVE ISOTOPES¹

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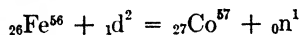
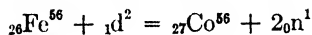
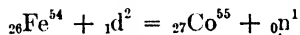
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The differential elimination of labeled inorganic elements in bile, pancreatic juice, and other secretions of the gastrointestinal tract has been under investigation in these laboratories. It was shown previously that intravenously injected radiosodium is rapidly excreted in both pancreatic juice (1) and bile (2). The concentrations of this labeled element in pancreatic juice resembled closely those in serum, whereas in the bile the levels of radiosodium were either the same as, or slightly higher than, those of plasma or serum. In the case of zinc, it was shown that the radioactive isotope of this element, when injected intravenously, is excreted in large amounts in the pancreatic juice, whereas very little is eliminated by way of the bile (3). The concentrations of radiozinc were much higher in the pancreatic juice than in the plasma, a finding that suggests that the acinar portion of this organ concentrates zinc.

In the present investigation, the elimination of radioactive isotopes of cobalt are compared in the external secretions of pancreas and liver of the dog. As pointed out previously (4), the sensitivity of the method for the measurement of radioactivity is such as to make possible the study of the excretion of these isotopes even when the amounts of labeled cobalt injected into the animal are too small to increase significantly the total cobalt content of the body.

EXPERIMENTAL. *Preparation of radioactive cobalt.* An iron target was subjected to a deuteron bombardment in the Berkeley cyclotron. Radioactive iron, cobalt, and manganese were thus produced. The nuclear reactions involved (5) in the production of the radiocobalt are shown below:



The half lives of Co^{55} , Co^{56} , and Co^{57} are 18.2 hours, 72 days, and 270 days respectively. By the time the radioactive cobalt was separated, the samples were always several weeks old; hence the radioactivity contributed by Co^{55} was negligible.

The surface of the iron target was dissolved in concentrated HCl and the major portion of the iron removed from this solution by extraction with ether. The iron that remained in solution was precipitated by the addition of KH_2PO_4 .

¹ Aided by a grant from the Christine Breon Fund for Medical Research.

and removed by filtration. This precipitate was redissolved in HCl, KH_2PO_4 was again added, and the resulting precipitate separated. The filtrates were combined and made slightly alkaline with NH_4OH , and the cobalt extracted with a chloroform solution of dithizone. The chloroform was evaporated and the residue ignited for approximately 12 hours at 400°C . The ash was dissolved in HCl and a few crystals of NaHSO_3 added to reduce higher oxides of cobalt which might have formed at the elevated temperature. The chloride and sulfite ions were precipitated by the addition of silver nitrate and removed by filtration. The filtrate was evaporated to dryness and the residue dissolved in 2 cc. of distilled water. The cobalt was then precipitated as silver potassium cobaltinitrite by the addition of 2 cc. AgNO_3 solution (1 per cent in 6N acetic acid) and 2 cc. 50 per cent KNO_2 . This precipitate was dissolved in concentrated HNO_3 and reprecipitated twice. The precipitate was then dissolved in concentrated HNO_3 . The silver ion was precipitated with HCl and the AgCl filtered out. In order to remove HCl and HNO_3 , the filtrate was evaporated to dryness, water added to the residue, and the mixture again evaporated to dryness. The quantity of cobalt present in the residue was measured colorimetrically by dissolving it in an alcoholic (95 per cent) solution of KSCN and comparing the resulting solution with suitable standards.

The alcoholic solution of radiocobalt was evaporated to dryness and the cobalt dissolved in dilute HCl. The solution was then made alkaline with NH_4OH and the cobalt extracted with a chloroform solution of dithizone. The cobalt was freed from the dithizone by evaporating the solution to dryness and igniting. The cobalt was dissolved in dilute HCl and evaporated to dryness to remove the excess HCl. The CoCl_2 was dissolved in such a volume of H_2O that the resulting solution contained 10 gammas of cobalt² per cc. The radioactive cobalt thus obtained as a mixture of Co^{56} and Co^{57} with half lives of 72 and 270 days respectively. Small aliquots were checked for the presence of radioactive iron or manganese contamination by adding salts of Co, Fe, and Mn as carriers and separating them by ordinary quantitative methods. The Fe and Mn fractions were found to be free of radioactivity.

Preparation and care of animals. Five dogs weighing between 16.5 and 19.3 kgm. were used. Dog I was prepared with a biliary and pancreatic fistula, dogs II, IV, and V with biliary fistulae, and dog III with a pancreatic fistula.

A modification of the Rous-McMaster procedure (6) for the preparation of the biliary fistula was used. The common bile duct was doubly ligated and divided between the ligatures. The gall bladder was then removed and the cystic duct cannulated with a glass cannula to which a rubber tube was attached. The rubber tube was brought out of the abdominal cavity through a stab wound. The intra-abdominal portions of the rubber tubing were wrapped in omentum to prevent kinking.

Pancreatic fistulae were prepared by a modification of the Elman-McCaughan

² A carrier in the form of stable cobalt was *not* added in the isolation of radiocobalt. The amounts of stable cobalt contained in this solution were present in the iron target as an impurity.

procedure as previously described (1). In dog I the 2 tubes were brought out through a single stab wound.

The dogs were fed twice daily. Each meal consisted of 200 to 300 grams of cooked meat supplemented with vitamin concentrates. Dogs with pancreatic fistulae received in addition 100 grams of raw pancreas twice daily, while those with bile fistulae were fed 5 grams of Bilron (Eli Lilly) with each meal. To compensate for the loss of fluids, each animal received intravenously 1000 to 2000 cc. of Ringer's solution daily.

For the first few days after the operation, the pancreatic juice and bile were drawn off once daily, measured, and discarded. Bile and pancreatic juice were withdrawn aseptically. When it became apparent that the secretions were being produced in adequate amounts, radiocobalt was administered about one half hour after the morning meal.

From 1.0 to 2.6 cc. of the cobalt solution were injected into the hind-leg veins of the dogs. Four to five samples of bile and pancreatic juice were obtained during the first hour; during the next 4 hours samples were removed at half-hour intervals. These samples were collected in 15 cc. centrifuge tubes. Later samples were collected in rubber bags. Blood was removed from the external jugular vein or from a vein of the foreleg.

Methods of analysis. One cubic centimeter samples of bile and pancreatic juice were transferred to rectangular dishes (2.5 x 6.0 cm.) pressed from thin sheets of aluminum foil. Lens paper was placed in the bottom of these dishes; this served to spread the liquid uniformly and to prevent splattering during the subsequent evaporation. After the evaporation, the sides of the dish were flattened, the entire aluminum foil with its contents wrapped in cellophane and sealed with scotch tape so that only one thickness of cellophane came between the dried material and the aluminum wall of the Geiger counter. Its radioactivity was determined after the manner described in a previous paper (1). The water content of these secretions was determined by evaporating aliquots to dryness at low temperatures in a vacuum oven.

Blood samples were heparinized, centrifuged for 5 minutes at 3600 R.P.M., and the plasma decanted. The radioactivity and water content of plasma were determined in the manner described above for pancreatic juice and bile.

The radioactivity of each sample was expressed as the percentage of the administered radioactivity recovered per cubic centimeter of water.

RESULTS. Pancreatic juice. The excretion of intravenously injected radiocobalt in pancreatic juice was measured in dogs I and III. The former had both pancreatic and biliary fistulae, whereas the latter had only a pancreatic fistula. The results are shown in figures 1 and 2. The excretion of radiocobalt in pancreatic juice was practically negligible. Thus in 70 hours after its injection into dog I (fig. 1) only 0.3 per cent of the injected radiocobalt appeared in the external secretion of the pancreas. Dog III excreted less than 0.1 per cent of the injected cobalt into the pancreatic juice in 48 hours. At no time did the concentration of radiocobalt in pancreatic juice approach that of plasma.

Bile. The excretion of radiocobalt in bile was determined in dogs I, II, IV, and V. Dogs II, IV, and V had biliary fistulae only, whereas dog I, as noted above, had both a biliary and a pancreatic fistula.

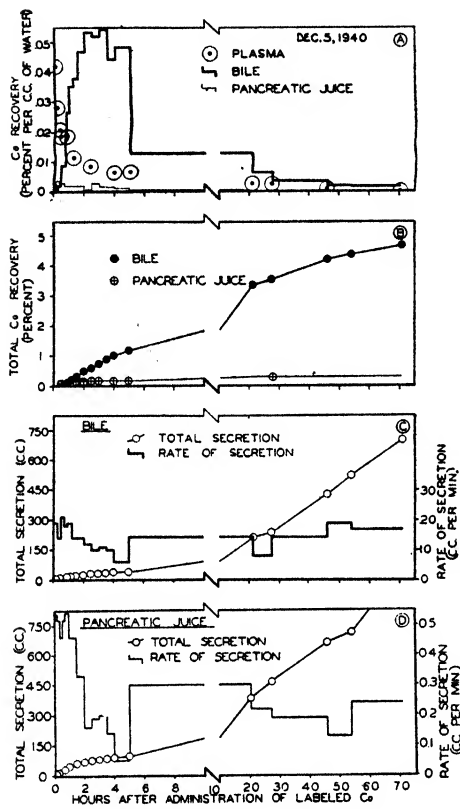


Fig. 1

Fig. 1. The elimination of intravenously injected radiocobalt in the pancreatic juice and bile of dog I.

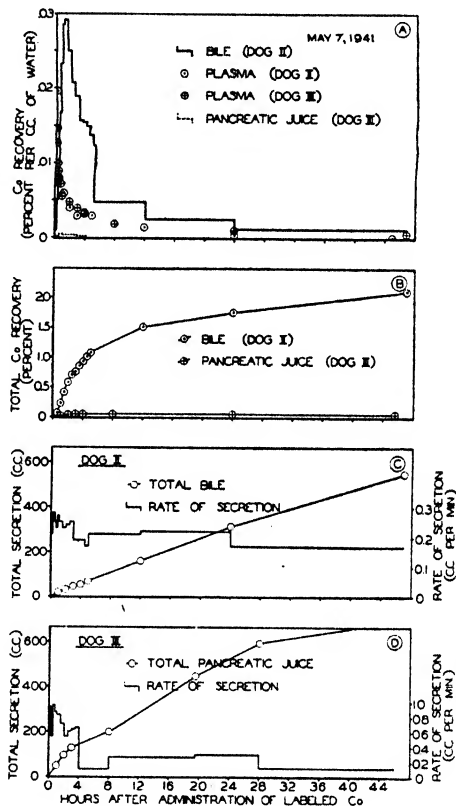


Fig. 2

Fig. 2. The elimination of intravenously injected radiocobalt in the bile of dog II and in the pancreatic juice of dog III.

After its intravenous injection, radiocobalt appeared early in the bile, and by one hour the concentration of radiocobalt was higher in bile than in plasma. The pronounced differences in the concentrations of radiocobalt in plasma and bile are well shown in figures 1 and 2. During the 42-54 minute interval after the introduction of radiocobalt, the bile of dog I contained an average of 0.27 per cent of the injected radiocobalt per cc. of water; at 51 min. the plasma contained 0.018 per cent per cc. of water. Between 2 and 2.5 hours the bile contained 0.054 per cent; at 2.3 hours the plasma contained 0.008 per cent. Although the

difference between the concentrations of radiocobalt in plasma and bile was more marked in the early than in the late periods, nevertheless the bile still contained a higher concentration of radiocobalt than the plasma as late as 72 hours.

Between 1 and 1.5 hours the bile of dog II contained 0.029 per cent of the injected radiocobalt per cubic centimeter of bile water, whereas at 1.2 hours the plasma contained 0.006 per cent (fig. 2). Between 4 and 4.5 hours, 0.015 per cent of the injected radiocobalt was recovered per cc. of bile water; at 3.9 hours the plasma of dog II contained 0.003 per cent per cc. Equally striking differences were observed in the concentrations of radiocobalt in plasma and bile of dogs IV and V. The last samples of plasma and bile were obtained from dogs II, IV, and V between 44 and 48 hours after the intravenous introduction of the radioisotopes, and at these intervals the concentration of the radiocobalt in the 2 fluids still differed considerably.

The significance of the liver in the metabolism of cobalt is well shown in the total amounts of radiocobalt eliminated in bile in 48 and 72 hours. In dog I a total of 4 per cent of the administered radiocobalt was recovered in the bile in 48 hours and 5 per cent in 72 hours. In 48 hours dogs II, IV and V excreted a total of 2 per cent by way of the bile.

DISCUSSION. The possibility that bile serves as a pathway for the elimination of cobalt was claimed as early as 1874 by Mayencon and Bergeret (7) and again in 1884 by Stuart (8). But it remained for Caujolle to establish definitely that parenterally administered cobalt makes its appearance in the bile of the dog (9). Then Greenberg *et al.*, with the aid of the radioactive isotopes of cobalt, were able to show that, although cobalt is excreted from the body mainly in the urine, a significant portion of it appears in the bile (10).

In the present investigation the rôles of 2 intestinal secretions in the metabolism of cobalt are compared, namely, bile and pancreatic juice. The finding that a significant portion of injected cobalt is eliminated in the bile is confirmed. As much as 5 per cent of the intravenously introduced cobalt was excreted in the bile by the end of 72 hours. The contrast between pancreatic juice and bile in their handling of this element is strikingly shown in the results presented here, particularly in the case of dog I, in which both pancreatic and biliary fistulae had been prepared. Practically none of the intravenously injected cobalt appeared in the pancreatic juice. Although the significance of these observations is not apparent at the present time, it is of interest to note that this difference between bile and pancreatic juice may reflect merely the permeability of the hepatic cell and the acinar cell of the pancreas to cobalt salts.

SUMMARY

Pancreatic juice and bile were compared as vehicles for the elimination of intravenously injected cobalt. The use of radioactive isotopes of this element permitted the introduction of as little as 10 to 26 gammas in dogs weighing 16 to 19 kgm. The dogs were provided with either a single bile fistula or a single pancreatic fistula or with both pancreatic and bile fistulae.

Significant amounts of cobalt were not eliminated in pancreatic juice. Ap-

preciable amounts did appear in the bile; a total of 5 per cent of the intravenously injected cobalt was contained in the bile collected during a period of approximately 72 hours.

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POTASSIUM DEFICIENCY IN THE RAT

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Studies of potassium deficiency in the rat have been made by a number of workers with conflicting results and interpretations. Osborne and Mendel (1) reported that rats fed diets containing 0.033 per cent potassium survived and grew as well as control rats fed diets containing 0.833 per cent potassium. Miller (2) was unable to confirm these findings and reported growth retardation when the dietary potassium content was reduced below 0.10 per cent. Leulier and Vanhems (3), Grijns (4) and Heppel and Schmidt (5) reported poor growth and early death in rats fed diets of low potassium content (0.0015 to 0.016 per cent). Schrader et al. (6) noted myocardial, renal and numerous other lesions in addition to growth failure and poor survival. Thomas et al. (7) also found myocardial lesions, poor growth and early death in rats fed diets containing approximately 0.02 per cent potassium. However, these workers postulated that this syndrome was dependent upon a concomitant vitamin B₆ deficiency. Orent-Keiles and McCollum (8) and Follis, Orent-Keiles and McCollum (9) with a diet containing 0.01 per cent potassium, observed a weight gain of 200 grams in 20 weeks and noted the development of myocardial and renal lesions. They attributed the findings of growth failure, widespread lesions and early death reported by some earlier workers to concurrent vitamin deficiencies. More recently, Follis (10) has reported failure of weight gain and early death in rats fed a diet containing only 0.001 per cent potassium. Skinner and McHargue (11) have observed similar results with a diet containing 0.005 per cent potassium.

The current availability of highly purified diets which permit good growth and the question whether or not vitamin deficiencies were responsible for some of the findings in earlier studies prompted us to reinvestigate the effects of potassium deficiency in the rat.

EXPERIMENTAL. Albino rats (Wistar or Osborne and Mendel) were weaned at about 22 days and within 1 week thereafter were placed on one of the experimental diets. The basal potassium-deficient diet no. 1007¹ contained 0.01 per

¹ Diet no. 1007 consisted of anhydrous dextrose 69.17 per cent, casein (leached and alcohol-extracted) 18.0 per cent, Crisco 8.0 per cent, and salt mixture (no. 21 of Orent-Keiles and McCollum, 1941) 4.83 per cent. Into 100 grams of this diet were incorporated 1 mgm. of thiamine hydrochloride, 2 mgm. of riboflavin, 1 mgm. of pyridoxine hydrochloride, 4 mgm. of calcium pantothenate, 2 mgm. of niacin, 200 mgm. of choline chloride, 0.001 mgm. of biotin and 0.4 mgm. of 2-methyl-1,4-naphthoquinone. Twice weekly each rat received a supplement of 0.25 cc. of corn oil containing 2000 units of vitamin A and 200 units of vitamin D (Natola) and once weekly 3 mgm. of α -tocopherol in 0.03 ethyl laurate.

cent potassium or less as determined² by the method of Shohl and Bennett (12) as modified by Fenn et al. (13). The potassium-containing diets were prepared by substituting the desired amount of potassium chloride for an equal weight of dextrose. The rats were weighed twice weekly. Some rats were allowed to die while others were sacrificed by decapitation or by exposure to illuminating gas. Gross autopsies were performed. The tissues were fixed in 3.8 per cent aqueous formaldehyde solution, embedded in paraffin and stained routinely with hemalum-azure eosinate and with iron hematoxylin-picrofuchsin. Frozen sections of liver and kidney were stained for fat. Heart, lungs, liver, pancreas, esophagus, large and small intestines, adrenals, kidneys, skeletal muscle, femur and thoracic spinal column were examined.

Production and pathology of potassium deficiency (Experiment I). In this experiment 17 rats of both sexes were fed the basal diet no. 1007 containing 0.01 per cent potassium. Seven rats were allowed to die and 10 were sacrificed when they appeared moribund. In subsequent experiments (II, III and IV) 23 rats were fed diet no. 1007 or diet no. 1033 also containing 0.01 per cent potassium and allowed to die. All of these 40 rats failed to gain weight, exhibited poor appetite, had an untidy appearance, and before death often became distended and had diarrhea. The average initial weight was 42 grams (range: 30 to 53 grams) and the average final weight was also 42 grams (range: 29 to 55 grams). The average survival time on the diet was 22 days (range: 11 to 37 days).

Gross and microscopic lesions were numerous and prominent and their incidence is given in table 1.

Grossly, hydrothorax and ascites were noted. The fluid was clear, yellow and in some cases of ascites in excess of 2 cc. The heart showed auricular and ventricular white and red spots. The lungs were congested. The small and large intestines showed dilatation with thin walls or they appeared swollen, pearly white and thick-walled. The involvement was often segmental with congestion in occasional segments. Intussusceptions were numerous. The pancreas appeared cystic. The kidneys were enlarged and had small, diffusely scattered purplish areas.

Microscopically, the heart showed auricular and ventricular myocardial necrosis followed by leucocytic infiltration and finally by lightly collagenized scars. Large auricular mural thrombi were seen occasionally with early lesions. The lungs showed diffuse congestion and in some cases edema. Pulmonary veins (which have cardiac muscle in the rat) occasionally showed lesions similar to those in the heart. The intestines showed dilatation or edema of the wall, especially of the submucosa. The muscularis showed swelling of the smooth muscle fibers which occasionally appeared hyalinized and intensely oxyphilic. Intestinal lymphoid follicles were atrophic and occasionally showed an increase of reticulo-endothelial cells. The pancreas showed striking separation of the lobules

² We are indebted to Dr. Herbert Tabor of the National Institute of Health for making this determination. A spectrographic analysis carried out through the kindness of Dr. L. T. Steadman of the University of Rochester indicated a potassium level in the basal diet of the order of 0.004 per cent or less.

presumably on the basis of interstitial edema, although no edema protein was evident. In the kidneys there were numerous amphophilic to basophilic tubular

TABLE 1
Incidence of lesions

EXP. NO.	DIET NO.	POTASSIUM CONTENT OF DIET	NO. OF RATS	NO. OF RATS DYING	SURVIVAL (AVERAGE)	NO. OF RATS WITH LESIONS							
						Heart	Kidney	Intestine	Pancreas	Spleen	Bone marrow	Ascites	Hydrothorax
I	1007	0.01	7	7	days 24	5	6	4	5	7	7	2	3
	1007	0.01	10	10†	22	8	6	6	6	10	9	2	10
II	1007	0.01	6	6	26	4	5	3	5	6	6	1	3
	$\left\{ \begin{array}{c} 1007 \\ \downarrow \\ 1025 \end{array} \right\}$	$\left\{ \begin{array}{c} 0.01 \\ \downarrow \\ 0.33 \end{array} \right\}$	6	0		0	0	0	0	0	0	0	0
	1025	0.33	6	0		0	0	0	0	0	0	0	0
	$\left\{ \begin{array}{c} 1025 \\ \downarrow \\ 1007 \end{array} \right\}$	$\left\{ \begin{array}{c} 0.33 \\ \downarrow \\ 0.01 \end{array} \right\}$	6	6	47	6	6	3	1	6	5	1	1
III	1033	0.01	8	8	26	4	5	7	6	8	8	3	4
	1034	0.33	8	0		0	0	0	0	0	0	0	0
IV	1007	0.01 *	9	9	15	4	3			9	9		
	1008	0.02	5	5	18	3	5			5	5		
	1009	0.03	5	5	20	2	3	3		5	5		
	1010	0.05	5	5	27	2	5	2		5	5		
	1023	0.09	5	0		2*	2†	0	0	0	0	0	0
	1024	0.17	5	0		1*	0	0	0	0	0	0	0
	1025	0.33	5	0		0	0	0	0	0	0	0	0
	1026	0.49	5	0		0	0	0	0	0	0	0	0
V	1009	0.03	5	5	24	4	5	2	3	5	5	0	5
	1010	0.05	6	4	23	2	4	3	2	6	6	0	1
	1023	0.09	6	0		4	4	0	0	0	0	0	0
	1041	0.13	6	0		0	0	0	0	0	0	0	0
	1024	0.17	6	0		0	0	0	0	0	0	0	0
	1042	0.21	6	0		0	0	0	0	0	0	0	0
	1043	0.25	6	0		0	0	0	0	0	0	0	0

* Minute myocardial scars.

† Regeneration of renal tubular epithelium.

‡ Sacrificed when moribund.

casts, irregular dilatation of collecting and convoluted tubules and fraying of the epithelium of distal convoluted tubules. No fatty degeneration was demonstrated. Glomeruli appeared normal. The adrenal cortex and liver occasionally

showed hydropic degeneration. In the spleen, marked congestion of the red pulp, absence of hematopoietic cells, and small inactive lymphoid follicles were noted. The bone marrow showed very marked congestion and marked reduction of hematopoietic elements. The remaining hematopoietic islands showed apparently normal proportions of the myeloid and erythroid series.

Blood counts by methods previously used (14) were made within 48 hours of death on 8 rats fed the basal, potassium-deficient diet (no. 1007). No leucopenias or anemias were noted. Average values were 11,075 for white blood cells per cu. mm. (range: 5,200 to 18,300), 4,650 for polymorphonuclear granulocytes per cu. mm. (range: 1,000 to 11,800) and 54 volumes per cent for hematocrit (range: 40 to 64).

Effects of potassium administration to potassium-deficient rats and of withdrawal of potassium from potassium-supplemented rats (Experiment II). Twenty-four male Osborne and Mendel rats were divided into 4 groups equal with respect to weight and litter distribution (fig. 1). Group A was fed the basal diet (no. 1007) containing 0.01 per cent potassium. Group B was fed the basal diet for 12 days and then fed the 0.33 per cent potassium-containing diet (no. 1025). Group C was fed diet no. 1025 containing 0.33 per cent potassium throughout the experiment. Group D was fed the 0.33 per cent potassium-containing diet (no. 1025) for 12 days and then fed the basal diet (no. 1007) containing only 0.01 per cent potassium. The growth data are in figure 1 and the incidence of lesions is in table 1. Rats in group A failed to grow and died after 26 days (average). Rats in group C gained approximately 3.8 grams daily, appeared healthy and showed no gross or microscopic lesions after 10 weeks on the diet. Rats in group B failed to grow while fed the 0.01 per cent potassium-containing diet (no. 1007) but when switched to diet no. 1025 containing 0.33 per cent potassium, growth started promptly and at a rate equal to that of rats in group C. Rats in group D, weighing 94 grams (average) after 12 days on diet no. 1025, when switched to diet no. 1007 ceased growing and survived an average of 47 days (range: 31 to 60 days) after starting diet no. 1007. During this period, 4 of the 6 rats lost weight (4, 8, 9 and 26 grams) and 2 rats gained weight (2 and 9 grams).

Food utilization in potassium-deficient rats. Lack of effect of increased vitamin intake on potassium-deficient rats (Experiment III). Sixteen Wistar rats of both sexes were divided into 2 groups equal with respect to sex, weight and litter distribution. Rats in group A were fed diet no. 1033³ containing 0.01 per cent potassium. This diet differed from the basal diet no. 1007 only in containing L. casei factor⁴ ("folic acid") (15), inositol, p-aminobenzoic acid and larger amounts

³ Diet no. 1033 was the same as diet no. 1007 except for its vitamin supplement. Into 100 grams of diet no. 1033 were incorporated 2.5 mgm. of thiamine hydrochloride, 4 mgm. of riboflavin, 2.5 mgm. of pyridoxine hydrochloride, 10 mgm. of calcium pantothenate, 5 mgm. of niacin, 300 mgm. of choline chloride, 0.01 mgm. of biotin, 1 mgm. of 2-methyl-1,4-naphthoquinone, 0.1 mgm. of L. casei factor, 2.5 mgm. of inositol and 2.5 mgm. of p-aminobenzoic acid. Separate supplements of vitamins A, D and E were administered as with diet no. 1007.

⁴ A crystalline fermentation product furnished through the courtesy of Dr. E. L. R. Stokstad and Dr. B. L. Hutchings of Lederle Laboratories, Inc.

FIGURE 1

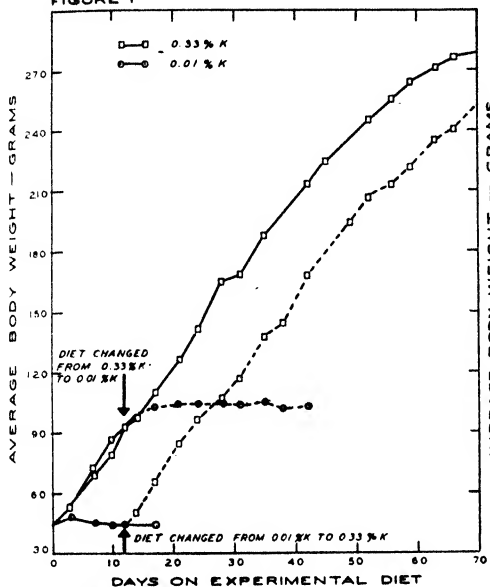


FIGURE 2

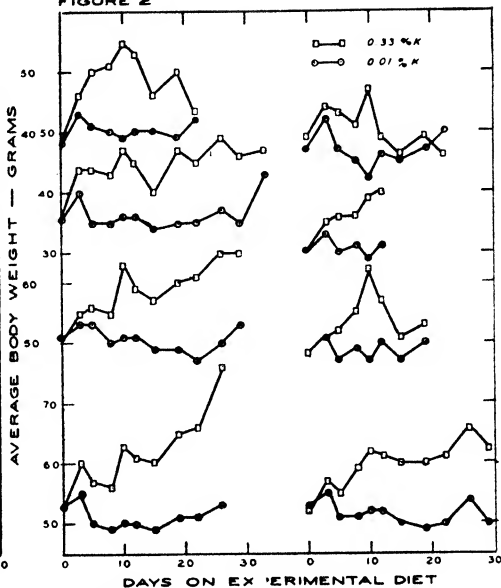


FIGURE 3

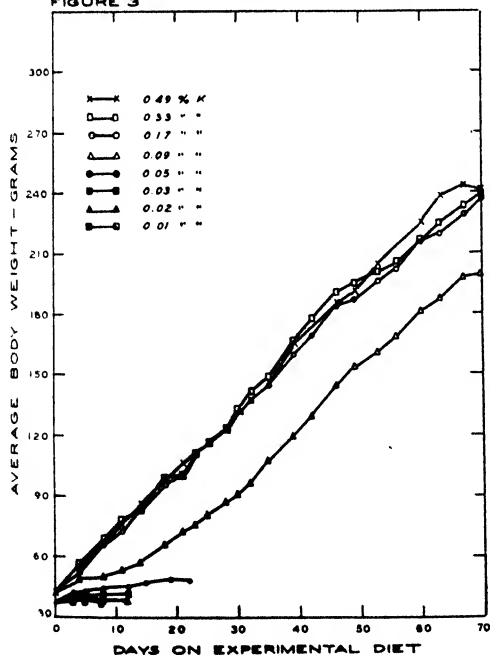
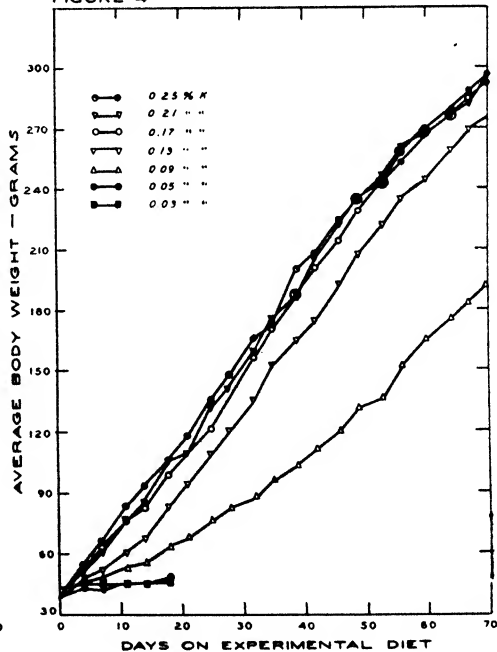


FIGURE 4



Figs. 1-4

of the other vitamins. Rats in group B were fed diet no. 1034 containing 0.33 per cent potassium but otherwise identical to the diet (no. 1033) fed rats in group A. Each rat in group B was pair-fed with its litter mate in group A. The growth curves for the 8 pairs of rats (fig. 2) demonstrate, in general, the better food utilization of the potassium-supplemented rats (group B). Rats in group A, despite their high vitamin intake as compared with rats fed the basal potassium-deficient diet no. 1007, died after an average of 26 days (range: 15 to 33 days) and developed lesions in high incidence (table 1). Rats in group B were fed ad libitum after the death of their potassium-deficient litter mates and were sacrificed after 30 days. During this period, the rats gained 3.0 grams per day and no lesions or residua of old lesions could be found grossly or histologically.

Potassium requirement for growth and prevention of lesions (Experiments IV, V). In experiment IV, male Wistar rats were fed diets containing 0.01, 0.02, 0.03, 0.05, 0.09, 0.17, 0.33 or 0.49 per cent potassium. In experiment V, male Osborne and Mendel rats were fed diets containing 0.03, 0.05, 0.09, 0.13, 0.17, 0.21 or 0.25 per cent potassium. The growth curves (figs. 3, 4), survival and incidence of lesions (table 1) indicate that at or near a dietary potassium level of 0.13 per cent, lesions were prevented and that at or near a dietary potassium level of 0.17 per cent weight gain was optimal. The average daily food intake per rat on diet no. 1024 containing 0.17 per cent potassium was 9.7 grams from the 1st to the 21st days and 13.6 grams from the 22nd to the 42nd days.

Discussion. Feeding a diet containing 0.01 to 0.05 per cent potassium to weanling rats has resulted in a failure of growth, severe, multiple lesions and early death. The findings of growth failure and poor survival are in essential agreement with the data of some previous workers (2-6, 10, 11) but are not in agreement with the findings of others (1, 8, 9). The occurrence of widespread edema and of lesions in sites other than the heart and kidney have been described by only one other group of investigators (6). Lesions of the adrenal cortex, pancreas, intestinal musculature and bone marrow have not been described previously.

Some investigators have considered the reported findings of growth failure, numerous lesions and poor survival to be a result of concurrent vitamin deficiencies (7) (8). However, it would appear unlikely that deficiencies of vitamins have exerted a significant influence in the present studies. Large amounts of the known factors, including biotin, L. casei factor ("folic acid"), p-aminobenzoic acid and inositol were included in some of the experimental diets. No effect on the development of the signs of potassium deficiency was observed when most of the vitamins were increased in quantity. Rats fed the basal, potassium-deficient diets, supplemented with adequate amounts of potassium, gained approximately 25 grams per week over a 10 week period, showed no signs of disease and were free from gross or microscopic lesions. In addition, rats fed diets supplemented with adequate amounts of potassium and pair-fed with litter mates fed the potassium-deficient diet were found to have better food utilization and no signs of potassium deficiency were demonstrable. It is significant, too, that the withdrawal of potassium from a rapidly growing rat resulted in a *prompt* cessation

of growth. Furthermore, the administration of potassium to a sick, stunted, potassium-deficient rat resulted in an *immediate* gain in weight and growth at a rate identical to that of rats fed the potassium-adequate diets from weaning.

Diets containing 0.01 to 0.05 per cent potassium failed to permit growth or uniform survival. With diets containing 0.09 per cent potassium, the rats survived but growth was suboptimal and occasional lesions were noted. A dietary potassium level of 0.17 per cent potassium⁵ appeared to be near the point at which lesions were uniformly prevented and at which the further addition of potassium failed to increase the rate of growth.

A minimum dietary content of potassium may be calculated which would be necessary in order to deposit a given weight of body tissue of normal potassium content at a given food intake. Thus, for a rat consuming 10 grams of food daily and growing at a rate of 4 grams per day, it would require a dietary level of 0.10 per cent potassium to furnish the 10 mgm. of potassium contained in 4 grams of normal body tissue (5). Since the utilization of potassium may not be complete even when potassium-deficient diets are fed (5) (8), then the dietary potassium levels would have to be in excess of 0.10 per cent. Calculations such as these make it appear plausible that levels in the neighborhood of 0.13 to 0.17 per cent potassium are minimal for the optimal weight gains noted in these studies. When similar calculations are made for rats consuming diets low in potassium and corrections are made for the subnormal potassium content of their tissues (5), the observed failures in growth are readily explained with potassium as the limiting factor rather than on the basis of deficiencies of hypothetical factors.

SUMMARY

Weanling rats fed highly-purified, potassium-deficient diets containing 0.01 to 0.05 per cent potassium failed to gain weight and died after approximately 22 days. Widespread edema and lesions in the heart, kidneys, intestines, spleen, adrenal cortex, liver and bone marrow were found in most potassium-deficient rats.

Administration of potassium to stunted, potassium-deficient rats resulted in immediate growth at a rate (approximately 25 grams per week) equal to that of rats fed potassium-adequate diets from weaning. Withdrawal of potassium from rapidly growing rats weighing approximately 100 grams resulted in a prompt cessation of growth, multiple lesions and death in about 47 days.

Food utilization was impaired in potassium-deficient rats as compared with pair-fed potassium-supplemented litter mates.

Vitamins, including biotin, L. casei factor ("folic acid"), inositol and p-amino-benzoic acid, administered in large amounts did not prevent the growth failure, widespread lesions and early death in rats fed potassium-deficient diets.

A dietary potassium level of approximately 0.17 per cent appeared to be minimal for optimal growth and the prevention of lesions.

⁵ Ben Dor (16) found a dietary-potassium level of 0.17 per cent to be minimal for optimal growth of chicks.

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VISUAL THRESHOLDS AS AN INDEX OF PHYSIOLOGICAL IMBALANCE DURING INSULIN HYPOGLYCEMIA

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In recent publications the present authors have reported studies of the behavior of differential visual thresholds during anoxia (1944) and its modification by the ingestion of glucose (1945). Since visual thresholds respond so strikingly to disturbances in oxidation caused by a lowering of the oxygen tension in the inspired air, one might expect similar changes when hypoglycemia interferes with the oxidative processes (see Discussion). McFarland and Forbes have reported studies of the effects of a lowering of the blood sugar level on dark adaptation. They found that hypoglycemia and anoxia produced similar changes in the absolute thresholds during dark adaptation. Furthermore, an excess of oxygen tended to counteract the effects of insulin. The present study confirms these findings and extends them to differential thresholds, of which absolute thresholds are a special case in which one of the contrasted intensities is zero. The rapidity of these effects in relation to the fall and rise of the blood sugar level is also demonstrated.

The use of visual intensity discrimination as a test of the above effects has many desirable features, such as high sensitivity to anoxia, precision of measurement, independence from the degree of effort exerted by the subject, and stability. These attributes make the test a useful tool with which to measure the effects of disturbances in oxidative metabolism.

METHODS. Differential thresholds for light intensity were measured with the discriminometer designed by Crozier and Holway. This instrument and its adaptation to these experiments have been described in an earlier publication (McFarland, Halperin and Niven, 1944). Essentially the procedure consisted of determining the threshold intensity (ΔI) of a square white test stimulus, subtending a visual angle of one degree. This stimulus was foveally fixated and had a duration of one-tenth second. It was superimposed on a uniformly illuminated, circular background subtending a visual angle of forty degrees. The intensity of the background could be set at any desired level, I . During the exposure of the test stimulus, the total light intensity of the square area was $I + \Delta I$, while the intensity of the surrounding field was I . The difference between these quantities when the square was just distinguishable, i.e., the differential threshold, was therefore ΔI .

¹ This research has been aided by a grant from the Lockheed Aircraft Corporation. Acknowledgment is also made to the Linde Air Products Company for the supplies of nitrogen and oxygen used in these experiments.

Capillary blood from the finger was analyzed for glucose by the method of Folin and Malmros, modified for the use of a photoelectric colorimeter.²

PROCEDURE. All experiments were carried out in the post-absorptive state, the subjects having fasted for at least ten hours. The subjects were four male students, 15 to 18 years of age, who had been trained thoroughly in making the required observations. They had no physical or ocular abnormalities.

Each test was preceded by exposure of the eye to a bright field (118,000,000 milliphotons³) for three minutes, followed by dark adaptation for ten minutes in order to establish uniform conditions. The field brightness, I , was then adjusted to the level indicated in the graphs, and threshold measurements were made after several minutes of adaptation to this intensity. All observations were made with the right eye. Each group of ten threshold measurements was taken in about three minutes, followed by a rest of two minutes.

Further details of general procedure are described in our earlier publication (1944). A description of the specific types of experiments in this study, together with the results, is presented below.

EXPERIMENTAL RESULTS. Three types of experiments were performed as follows:

A. The effect of progressively increasing amounts of insulin on visual intensity discrimination at a constant, low brightness level. These tests were designed to demonstrate the magnitude of the visual response to insulin hypoglycemia as a function of time and in relation to the amount of insulin and the blood sugar levels. The results for three subjects are presented in figures 1, 2, and 3. Each curve in these figures represents a complete experiment carried out on a different day. Each point represents the mean of a group of ten threshold measurements. The vertical line through each point represents plus and minus the standard deviation of these ten measurements (in logarithmic units). The standard deviations were determined by the formula $\sigma = \sqrt{\sum d^2 / (n - 1)}$, where d is the deviation of each measurement from the mean and n is the number of measurements. The numbers below each curve represent the blood sugar levels (in mgm. per 100 ml.) at the times indicated by the arrows.

A constant background intensity producing a retinal illumination of 229 milliphotons ($\log_{10} I = 2.360$) was employed for these threshold measurements. This value corresponds roughly to the illumination produced by dim moonlight. It is shown below that this low intensity lies in the range in which intensity discrimination is most markedly affected by hypoglycemia, just as it is by hypoxic anoxia.

² The modification consisted of replacing the sodium carbonate-cyanide solution by 8 grams of anhydrous sodium carbonate made up to 500 ml., and substituting 1 ml. of potassium ferricyanide for the 2 ml. used in the visual colorimetric procedure.

³ Intensity of retinal illumination is measured in photons $\left(\text{millilamberts} \times \frac{10}{\pi} \times \text{pupil area in square millimeters} \right)$. In order to avoid negative logarithms in dealing with very low intensities, they are here expressed in units one-thousandth as large (milliphotons).

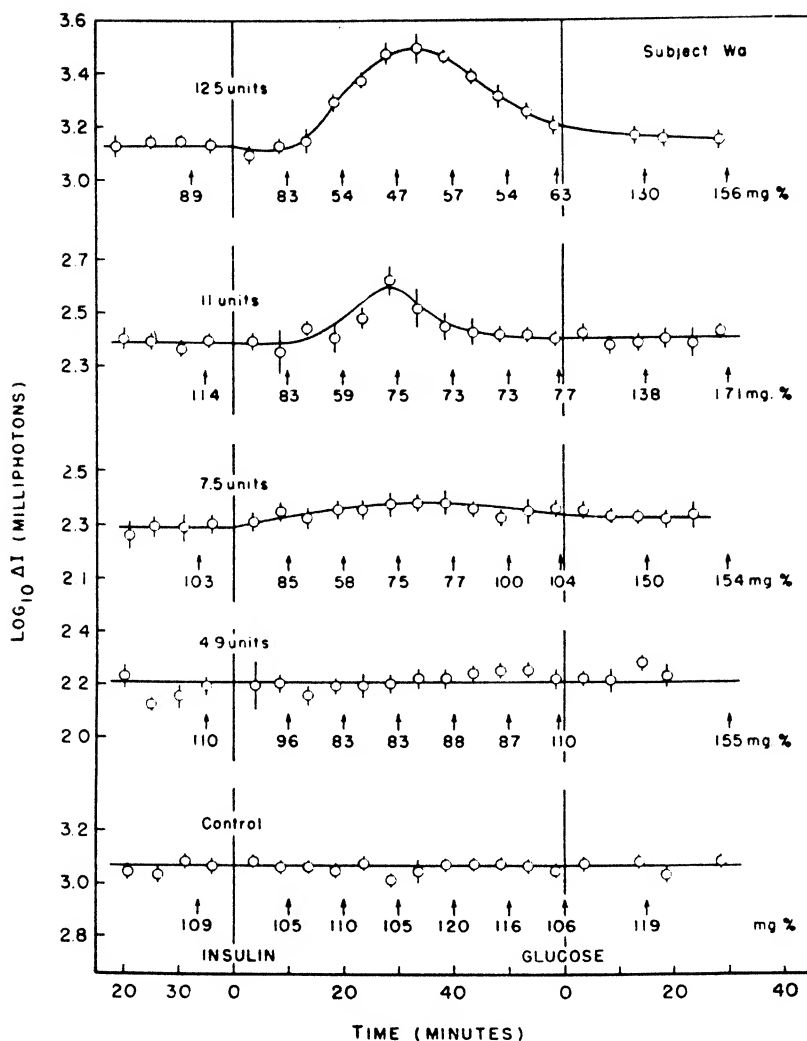


Fig. 1. The effect of progressively increasing amounts of insulin on visual intensity discrimination at a constant, low brightness level. The logarithms of the differential thresholds (ΔI) are plotted against time. A rise of the curves signifies impairment of sensitivity after intravenous injection of insulin in the doses indicated. The numbers below the curves represent the blood sugar levels at the indicated times. The length of the vertical line through each point denotes plus and minus the standard deviation of the group of ten measurements represented by the point. $\text{Log}_{10} I = 2.360$. Subject Wa.

These experiments were carried out as follows. After the preliminary light and dark adaptation, several groups of measurements of the differential thresholds were made until constant values were obtained. During this time a blood

sample was taken for sugar determination. Crystalline insulin in amounts as indicated on the graphs was then injected intravenously, starting with about 0.05 unit per kgm. body weight. Sterile, isotonic saline was used as a control. Thereafter, two-minute rest periods alternated with three-minute periods

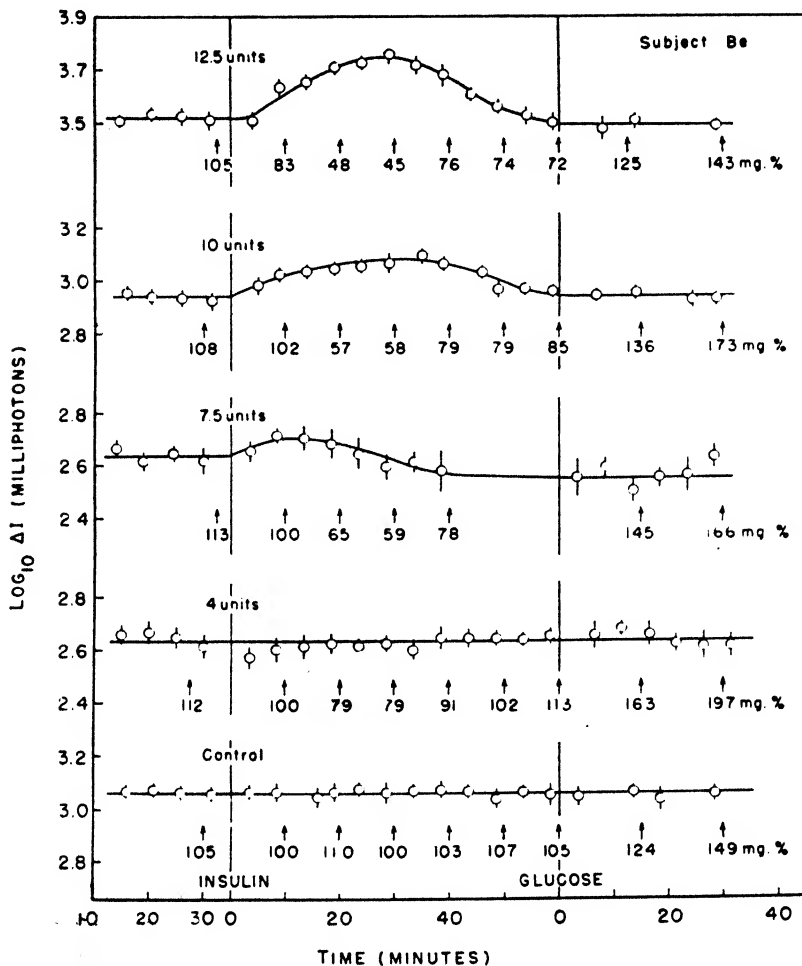


Fig. 2. See legend for figure 1. Subject Be.

during which threshold measurements were made. Blood samples were taken about every ten minutes. One hour after the administration of insulin, a solution of 50 grams of glucose in 250 ml. of water was given orally. The above procedure was continued for another half hour.

Figures 1 and 2, presenting the data for subjects Wa and Be, show that

during the control experiments as well as with the lowest amount of insulin there was no change in visual sensitivity. Thereafter, with increasing amounts of insulin which caused a drop of the blood sugar to lower levels, progressively greater elevations of the differential threshold occurred. The maximum effect

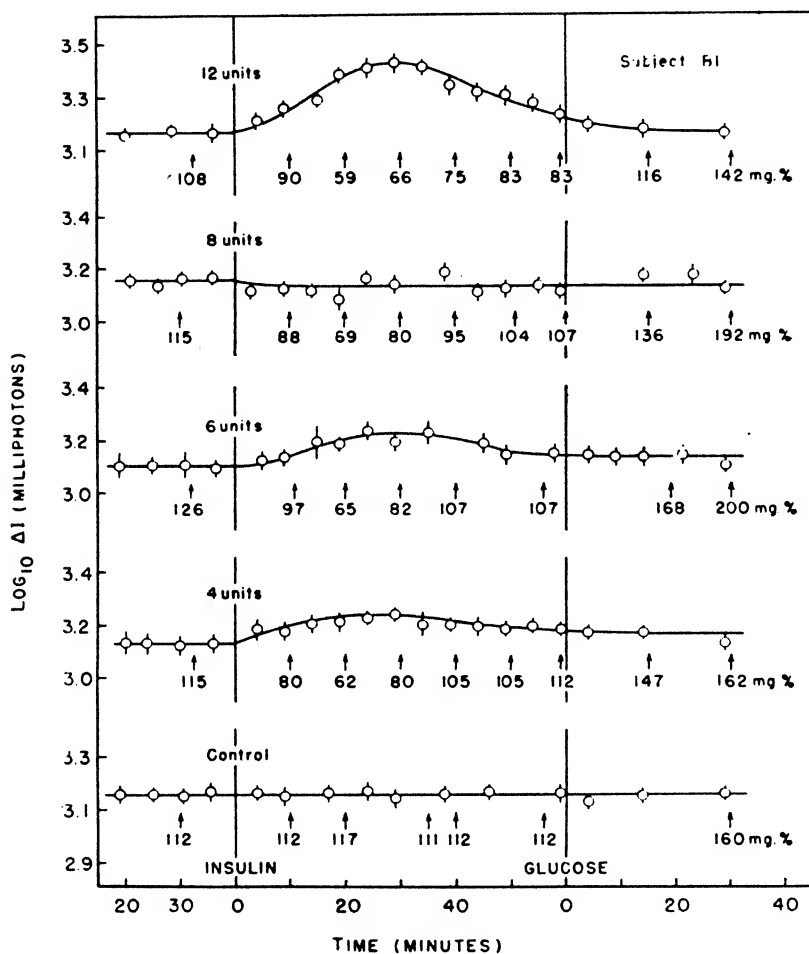


Fig. 3. See legend for figure 1. Subject Bl.

usually was observed about one-half hour after the intravenous injection of insulin, often occurring somewhat later than the time the minimum blood sugar level was reached. With the spontaneous recovery of the blood sugar, the visual thresholds again fell to levels at or near the initial measurements.

Figure 3, presenting the data for subject Bl, indicates a rise of the thresholds after 4 and 6 units of insulin, but not after 8 units. This is consistent with the

fact that in the latter experiment the blood sugar did not fall as low as in the two preceding experiments. In general, the data for these three subjects indicate that hypoglycemia induced by intravenous insulin results in an impairment of visual sensitivity only if the blood sugar level reaches a minimum of about 65 to 70 mgm. per 100 ml., or lower, as determined by the Folin-Malmros method.

Experiments were also made on a fourth subject (Ha). This individual showed a drop of his blood sugar to 58 mgm. per 100 ml. after only 4.5 units of insulin (0.05 unit per kgm. body weight). His visual thresholds rose about 0.5 log₁₀ unit. Thus the visual response is consistent with the changes in blood sugar despite the variability in insulin tolerance known to be present from person to person, or even in the same person from day to day.

The administration of glucose, one hour after insulin, resulted in an elevation of the blood sugar well above the fasting level. The effect of this hyperglycemia on the visual thresholds was merely to restore them to their original level in those instances in which spontaneous recovery was incomplete. At no time was visual sensitivity improved beyond that found during the fasting state. Comparable observations were made in an earlier publication by the present authors (1945), in which it was found that hyperglycemia partially counteracted the visual impairment caused by anoxia, but had no effect if the subject's visual sensitivity was not previously decreased.

B. *The effect of oxygen on the impairment of visual sensitivity by insulin.* This group of experiments was carried out in the same manner as above, with the exception that 100 per cent oxygen was administered for several minutes while the effect of insulin was maximum. The subjects wore a closely fitting rubber oro-nasal mask throughout the experiment, breathing room air through the mask while oxygen was not being administered. They were not aware of changes in the composition of the inspired air during the experiment.

The results of these tests are shown in figure 4. About one-half hour after the intravenous injection of 12 to 14 units of crystalline insulin, each subject showed a considerable rise of his visual threshold. At this time 100 per cent oxygen was administered. Within a few minutes the visual thresholds began to decline toward normal. The oxygen was continued only long enough to perform two or three groups of measurements, after which room air was restored. Within a few minutes the thresholds rose again, falling on the broken line which represents the expected curve without oxygen. The succeeding measurements continued to follow this curve. The second rise of the thresholds after discontinuance of oxygen shows clearly that the preceding drop was not due to spontaneous recovery; in other words, the effect of the insulin had not worn off.

When 100 per cent oxygen was given either before the insulin or after complete restoration with glucose, it had no effect on the unimpaired thresholds.

C. *The effect of insulin hypoglycemia on foveal differential thresholds as a function of light intensity.* The procedure followed in this series of experiments was similar to that in an earlier report (1944) dealing with the effects of anoxia on this relationship. After preliminary light and dark adaptation, differential thresholds were determined at each of several background intensities, over a

range of more than five \log_{10} units, in the order of increasing intensities. The combined light and dark adaptation was then repeated. Crystalline insulin was injected intravenously in amounts previously observed to cause marked threshold elevations at low intensities. Repeated measurements of the differential thresholds were made at the lowest intensity until about 25 to 35 minutes after the injection of insulin, when a marked elevation was observed. These

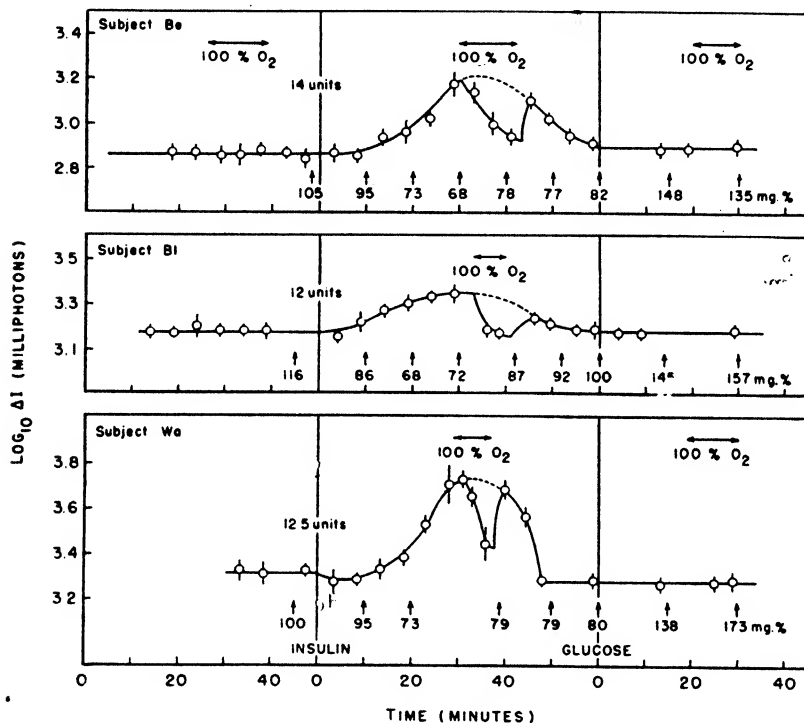


Fig. 4. The effect of oxygen on the impairment of visual sensitivity caused by insulin. The various symbols have the same meaning as in the preceding figures.

The administration of 100 per cent oxygen at the height of the effect of insulin restores the thresholds to nearly normal levels. Reversion to room air results in a rise of the curve, showing that the insulin was still exerting an effect. Oxygen does not alter the thresholds at fasting blood sugar levels, or after glucose.

were immediately followed by single groups of measurements at an intermediate and a high intensity. Only three intensities were used to establish the position of the curve during hypoglycemia, because it was necessary to make all these observations during a period of about 12 minutes before a significant amount of recovery took place. Upon the completion of these readings, glucose (50 grams in 250 ml. of water) was given orally. Light and dark adaptation was repeated, and one-half hour later measurements were again made at one or more intensities.

The results are shown in figure 5, where the Weber fraction $\Delta I/I$ is plotted against I on a double logarithmic scale. The open circles, representing the measurements before insulin, are fitted by a theoretical curve derived from Hecht's equation $\frac{\Delta I}{I} = C \left[1 + \frac{1}{(KI)^{1/2}} \right]^2$. This same curve was transposed horizontally to the right until it fell on the solid circles, representing the observations during hypoglycemia. A satisfactory fit was obtained. A similar shift of the intensity discrimination curve to the right has also been demonstrated during hypoxic anoxia (McFarland, Halperin and Niven, 1944). This shift of the curve means that in order to reach any given level of intensity discrimination ($\Delta I/I$), the background intensity must be multiplied by a constant factor greater than unity. The rise in $\log_{10} \Delta I$ (or $\log_{10} \Delta I/I$, the rise in both

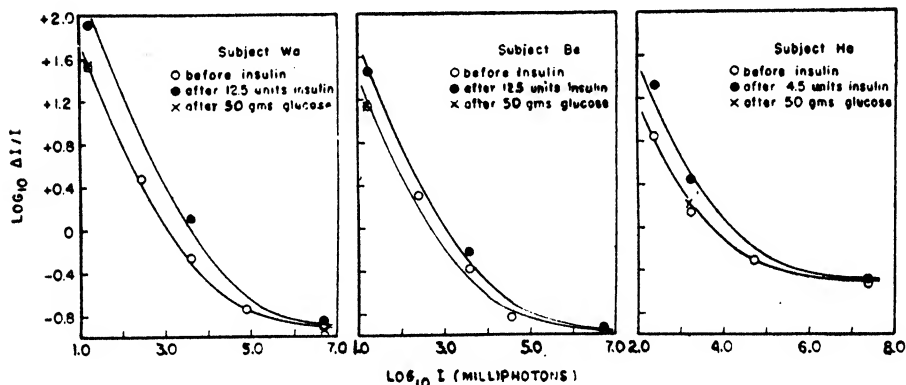


Fig. 5. The effect of insulin hypoglycemia on the relation between differential threshold⁸ and light intensity. The Weber fraction $\Delta I/I$ is plotted against intensity on a double logarithmic scale. The points represent actual observations. The curves are derived from Hecht's theoretical equation (see text). Hypoglycemia results in a transposition of the curve to the right, on the intensity axis.

being equal at a given value of I) varies with I . It is greatest where the curve is steepest, at the low intensities. As higher intensities are approached and the curve levels off, a shift to the right produces very little elevation of the threshold.

The points indicated by the symbol \times show that after glucose was ingested, the thresholds returned to their initial values.

A statistical analysis of the observed changes shown in figure 5 is presented in table 1. The critical ratios are the ratios of the observed changes to their standard errors. They are greatest at the lowest light intensities, where the largest threshold changes were observed. P represents the probability that a rise in the threshold might be due to chance. Values of P less than 0.05 are considered to indicate significant differences, and those below 0.01 are interpreted as highly significant statistically. The changes observed at the low and

intermediate intensities exhibit very high statistical significance for all three subjects. The change at the highest brightness level was not significant in two of the three cases, and in the third the critical ratio was relatively low.

Blood samples for sugar determination were taken every ten minutes after insulin, but were not simultaneous with the visual measurements. Values of the blood sugar at the time the visual measurements took place were estimated by graphic interpolation. These are shown in table 1. It may be noted that by the time the measurements were made at the highest light intensity, the blood sugar was already rising. It might therefore be contended that the lack of a significant rise in threshold at this intensity may be attributed to the fact

TABLE 1

The effect of insulin hypoglycemia on the relation between differential light thresholds and intensity

	LOG ₁₀ (I, IN MILLI- PHOTONS)	BEFORE INSULIN			AFTER INSULIN				RISE IN THRESH- OLD AFTER INSULIN	CRITI- CAL RATIO	P
		Log ₁₀ (ΔI, in milli- photons)	σ	Blood sugar	Log ₁₀ (ΔI, in milli- photons)	σ	Time after insulin	Blood sugar			
			log ₁₀ units	mgm. %		log ₁₀ units	min- utes	mgm. %	log ₁₀ units		
Subject Wa (12.5 units of insulin i.v.)	1.225	2.756	0.026	108	3.164	0.047	23.5	63	0.408	24.0	<0.01
	2.360	2.838	0.049								
	3.578	3.323	0.043		3.674	0.030	27.5	58	0.351	20.7	<0.01
	4.564	3.830	0.014								
	6.685	5.771	0.031		5.822	0.033	35.5	63	0.051	3.6	<0.01
Subject Be (12.5 units of insulin i.v.)	1.225	2.366	0.041	103	2.693	0.043	34.5	55	0.327	17.4	<0.01
	2.360	2.666	0.029								
	3.578	3.150	0.033		3.270	0.025	39.5	75	0.120	9.2	<0.01
	4.564	3.731	0.052								
	6.685	5.726	0.068		5.737	0.056	46.5	80	0.011	0.4	0.70
Subject Ha (4.5 units of insulin i.v.)	2.360	3.208	0.024	107	3.688	0.031	24.0	68	0.480	38.7	<0.01
	3.248	3.396	0.023		3.680	0.043	29.5	72	0.284	18.4	<0.01
	4.694	4.391	0.030								
	7.307	6.804	0.024		6.823	0.030	37.5	80	0.019	1.6	0.15

that the effect of the insulin was already wearing off. The following experiments indicate that this is not true.

The experiments shown graphically in figure 6 were designed to demonstrate how much the differential threshold rises at the highest light intensity ($\log_{10} I = 6.685$) at the time when insulin exerts its *maximal* effect. The latter is judged by the response of the threshold at a low brightness level ($\log_{10} I = 2.360$), at which it is quite sensitive to insulin. This intensity is high enough, however, so that the subject can re-adapt to it rapidly after exposure to the high intensity.

The upper curve in each graph of figure 6 represents measurements at the lower intensity; the other, measurements at the high brightness level. The solid

portion in each case denotes the time during which the eye was exposed to the indicated brightness level, while the broken portion represents the time during which the other intensity was employed.

After obtaining threshold measurements at each brightness level in the fasting state, 12.5 units of crystalline insulin were injected intravenously. Thresholds at the low intensity were then followed as in the first group of experiments de-

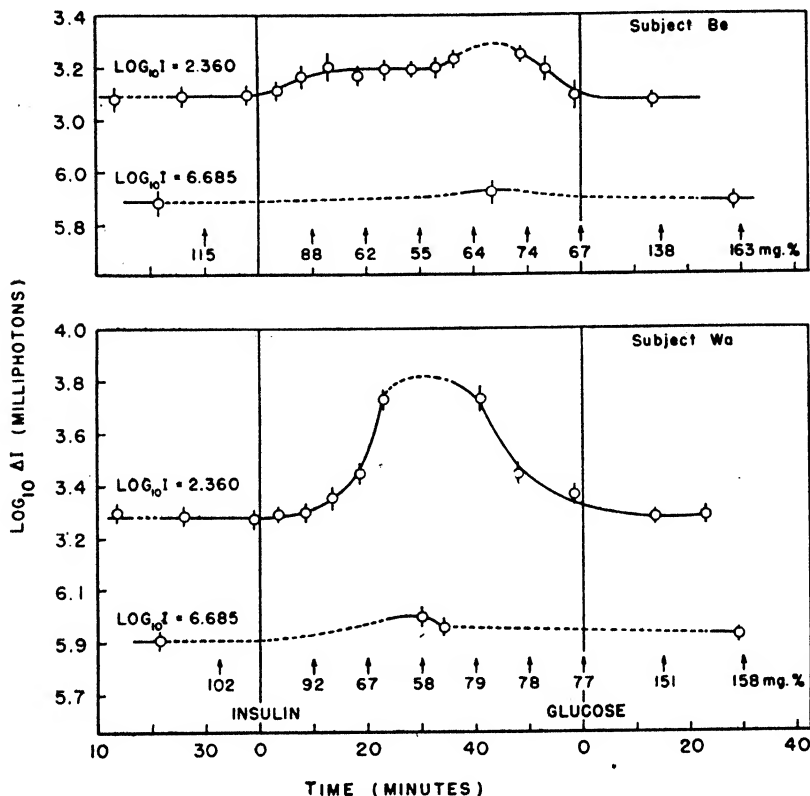


Fig. 6. The response of differential thresholds, at a high light intensity, to hypoglycemia. These thresholds, shown by the lower curve of each graph, are altered to only a slight degree even at the time when the thresholds at a low light intensity ($\log_{10} I = 2.360$) show a maximal response to hypoglycemia.

scribed above (A), for about 30 minutes, when a considerable elevation was reached. The high intensity was then presented, and after several minutes of adaptation, one or two groups of threshold measurements were made. Only a slight elevation of the threshold at this brightness was noted; this was of the same order of magnitude as is found in figure 5. Immediately thereafter, the low intensity was restored. After several minutes of adaptation to this bright-

ness, a time previously found to be sufficient to yield a constant threshold, measurements were resumed. These showed that the effect of the insulin had not begun to wear off. The results indicate that the differential thresholds at high brightness levels are only slightly affected by hypoglycemia, and confirm the validity of the results shown in figure 5.

DISCUSSION. The functioning of the central nervous system depends on a continuous and adequate supply not only of oxygen, but also of glucose. Apparently the sole source of energy which can be utilized by the nervous tissues is glucose (Himwich; Long; Wortis and Goldfarb). Even in totally depancrea-tized animals the brain always oxidizes carbohydrate, regardless of the impairment of carbohydrate metabolism in the remainder of the body (Himwich and Nahum). It therefore appears that the nerve cells require only two extrinsic substances for their energy metabolism, namely, oxygen and glucose. The cerebral supply of reserve carbohydrate in the form of glycogen is very limited (Kerr and Ghantus). The brain is therefore dependent largely on a continuous supply of sugar in the blood to satisfy its requirements. Reduction of the latter by such measures as the administration of insulin first causes impairment of cerebral function, followed by more serious disturbances manifested by convulsions and culminating in death. In a general way, these effects are similar to those of oxygen deprivation. It seems reasonable to believe that both anoxemia and hypoglycemia produce their effects in essentially the same way namely, by slowing the oxidative processes. Numerous workers⁴ have produced evidence suggesting strongly that as the blood glucose is decreased by insulin, the oxygen consumption of the brain declines along with its glucose utilization.

The study of vision under conditions of physiological imbalance is important, not only *per se* but also because certain visual functions are believed to reflect changes in the central nervous system, of which the retina is essentially a part both embryologically and anatomically. As yet the locus of the depressant effect of anoxia on visual sensitivity has not been definitely determined. Although the photochemical mechanism does not seem to be involved, either the neural elements of the retina itself or the geniculo-striate-cortical system of the brain may be affected. In the latter case, a change in visual thresholds may be considered with even more certainty to be an index of alterations in the function of the brain. A striking parallelism between changes in vision and in the electroencephalogram during anoxia and hypercapnia has been demonstrated by Gellhorn and Hailman. These authors reported studies in which electroencephalograms and the critical fusion frequency of flickering light were both recorded during the same experiments. They found that conditions of anoxia leading to a decline in critical fusion frequency are accompanied by typical anoxic changes in the brain as demonstrated by the EEG. Degrees of anoxia which did not alter the critical fusion frequency likewise had no effect on the EEG. Addition of 3 per cent carbon dioxide to oxygen-nitrogen mixtures greatly alleviated or

⁴ See Himwich and Fazekas; Dameshek and Myerson; Himwich, Hadidian, Fazekas and Hoagland; Loman; Schmidt, Kety and Pennes; Loman and Myerson; Himwich, Frostig, Fazekas and Hadidian.

completely offset the effects of anoxia on both the EEG and critical fusion frequency.

No simultaneous measurements of visual and electroencephalographic changes during alterations in blood sugar have been reported, to our knowledge. The results of various studies of the EEG during insulin hypoglycemia (Lennox, Gibbs and Gibbs; Gibbs, Williams and Gibbs; Davis; Brazier, Finesinger and Schwab) seem to show a striking parallelism with the findings of the present investigation. The most recent of these studies, by Brazier and her collaborators, was carried out on 45 subjects. Insulin, in doses of 0.10 unit per kgm. ideal body weight, was given intravenously. It was found that blood sugar levels below 70 mgm. per 100 ml., but insufficiently low to impair consciousness, may produce changes in the occipital potentials. In the present study, visual changes were observed at blood sugar levels below about 65 to 70 mgm. per 100 ml. The electroencephalographic changes consist of 1, slowing of the dominant frequency, and 2, development of activity slower than alpha. As in the case of visual thresholds, these EEG changes resemble those of anoxia. High blood sugar levels (above 130 mgm. per 100 ml.), induced by the ingestion of 100 grams of glucose, did not affect the electroencephalogram. We found no visual changes under similar conditions. In experiments on anesthetized cats, Gellhorn and Kessler found that the effects of hypoglycemia on the EEG can be completely offset by the inhalation of pure oxygen, provided that the hypoglycemia is not so severe as to produce almost complete cessation of brain function. This finding is also in accord with our observations on visual sensitivity. Davis noted that the EEG remained unstable for about 10 to 20 minutes after the blood sugar began to rise, while Himwich, Hadidian, Fazekas and Hoagland also found that EEG changes tended to lag behind the blood sugar curve, correlating more closely with the oxygen uptake of the brain. In the present study, a tendency was also noted for the maximum visual effect to occur somewhat later than the minimum blood sugar level. In all these details therefore, the visual alterations appear to follow a course strikingly parallel to the changes in cerebral activity.

An investigation of the foveal intensity discrimination function during anoxia by the present authors (1944) showed that the $\log_{10} \Delta I/I$ vs. $\log_{10} I$ curve is translated to the right. This translation has no vertical component. When these results were analyzed in terms of Hecht's theoretical equation $\frac{\Delta I}{I} =$

$C \left[1 + \frac{1}{(KI)^{1/2}} \right]^2$, the effect of oxygen deprivation was seen to be a decrease in the magnitude of the constant K . The value of C remained unaltered. The present study showed the effect of hypoglycemia to be identical with that of anoxia.

The analysis may be extended further in an attempt to determine what mechanism may account for a change in K . The above equation is a simplified form of the expression $\frac{\Delta I}{I} = \frac{c}{a^2 k_2} \left[1 + \frac{1}{[(k_1/k_2)I]^{1/2}} \right]^2$, where c is the increment of photo-products which must be formed in a given time in order to elicit a visual response,

a is the initial concentration of the photosensitive substance, and k_1 and k_2 are the velocity constants of the decomposition and regeneration reactions, respectively. Of these, anoxia or hypoglycemia might alter the value of c , k_1 , or k_2 , or any two or all of these. An increase in the value of c would cause an upward displacement of the $\log \Delta I/I$ vs. $\log I$ curve by increasing the value of C . A decrease in k_1 would cause a shift to the right by decreasing the value of K . An increase in k_2 would cause a shift to the right as well as a downward displacement by increasing K and decreasing C . Obviously, therefore, the observed change can be accounted for by a decrease in k_1 , the velocity constant for the decomposition of the visual pigment, but not by a change in any other single constant.

The same conclusion may be reached as follows. A change in the value of $K (= k_1/k_2)$ necessitates the assumption that either k_1 or k_2 , or both, must change. Since $C (= c/a_2k_2)$ is not affected, one must assume that any change in k_2 must be exactly compensated by an equal change in c . This would be an unlikely situation. Therefore, the simpler assumption would be a change in k_1 .

Such a change, i.e., a decrease in k_1 , might be interpreted as follows. Visual pigments are broken down less rapidly to form the photoproducts which are necessary to produce a visual sensation. Thus a greater intensity of light is needed to produce a visual response during anoxia or hypoglycemia.

Several other facts, however, must be taken into account. 1 It is well known that primary photochemical reactions are not affected by such factors as oxygen tension, temperature, and the like. Chase and Hagan showed that the kinetics of decomposition of visual purple *in vitro* is not altered in the complete absence of oxygen. They state, however, that the reactions *in vitro* may not be completely identical with those *in vivo*. 2. Hypoglycemia, produced by insulin, has the same effect as anoxia. There is no evidence that glucose is involved in the degeneration of visual pigments by light. Its rôle might be explained, however, in terms of central nervous tissue metabolism. 3. The change in foveal visual thresholds during anoxia is equal to that of peripheral thresholds. Since different photosensitive pigments are involved in cone and rod vision, it would seem that the velocity constants for at least two and possibly several reactions are affected to the same extent by anoxia. This fact would seem to be highly coincidental.

Because of these facts it is evident that the visual changes during hypoglycemia and anoxia cannot be explained in terms of Hecht's equation in its original form. The initial derivation of this equation was concerned with the simple retinal events which seem to be the determining factors of vision under normal conditions. During anoxia and hypoglycemia, factors which are normally secondary or constant (e.g., synaptic resistance between the neural elements of the retina and brain) may be involved to such an extent as to become limiting. Thus a factor which was implicit in the original equation needs to be brought out and developed in order to take into account the present findings. The following interpretation has been suggested by Hecht (personal communication).

The velocity constant k_1 is not a simple constant, even on the chemical level.

It must include elements such as an absorption coefficient, a dimensional constant and a real kinetic constant. Since the data are visual ones, k_1 probably also includes some factor which translates the photochemical processes into vision. This factor might be called an "effectiveness constant" since it would determine the central or nervous effectiveness of the photoproducts. Under normal or constant conditions, all the above constants remain unaltered, so that the whole complex can be represented by one constant k_1 . When, however, anoxia or other factors alter the situation central to the photochemical system, the effectiveness constant may well decrease. More light would then become necessary to produce the same *physiological* end product as before. As a result, the complex constant k_1 would be decreased.

In deriving the equation, this effectiveness factor may be represented explicitly as an additional constant, say e . The basic equation for the kinetics of decomposition of the visual pigment would then become $dx/dt = ek_1I(a - x)^m$, where x is the instantaneous concentration of photoproducts, m is the order of the reaction, and the other symbols are as above. The derivation of Hecht's stationary state equation would proceed as in his original publication (1934), and the effectiveness constant finally appears in the KI term as follows: $\frac{\Delta I}{I} = C \left[1 + \frac{1}{(eKI)^{1/2}} \right]^2$. The effect of anoxia and of hypoglycemia would then be a decrease in e rather than in K , which deals with the photochemical equilibrium.

SUMMARY

1. The changes in foveal intensity discrimination thresholds caused by insulin hypoglycemia were investigated on four human subjects. The insulin was administered intravenously in amounts varying from 4 to 14 units.

2. A rise in the visual thresholds (i.e., a decrease in visual sensitivity) is produced if the capillary blood sugar drops below about 65 to 70 mgm. per cent (Folin-Malmros method). The curve relating visual thresholds to time parallels the drop in blood sugar. The maximum effect occurs about 20 to 30 minutes after the injection of insulin, and then spontaneous recovery takes place.

3. The inhalation of 100 per cent oxygen results in a reversal of a large part of the impairment caused by hypoglycemia.

4. Hypoglycemia causes a translation of the curve relating $\log \Delta I/I$ to $\log I$ toward the right on the intensity axis. This shift is identical with the effect of low oxygen tensions. As a result of the shape of the curve, this means that the greatest effect occurs at low illuminations, and as the brightness is increased the effect becomes less.

5. The effect of hypoglycemia on vision is probably due to an impairment of the oxidative processes in the nervous tissues.

6. The interpretation of these findings in terms of Hecht's photochemical theory necessitates the introduction of a new "effectiveness" factor into his equations.

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EFFECTS OF ACUTE HEMORRHAGIC AND TRAUMATIC SHOCK ON RENAL FUNCTION OF DOGS^{1, 2}

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CRITIQUE OF PROCEDURES USED FOR ESTIMATION OF RENAL BLOOD FLOW AND FILTRATION. a. *Estimation of renal blood flow from excretion rate and whole blood A - R difference of an excretory substance.* Measurements of renal blood and plasma flow have been made as described by Van Slyke, Rhoads, Miller and Alving (1) by dividing the amount of an excretory substance excreted per minute by the amount which the kidneys extract from one volume unit of blood or plasma. The principle is similar to that applied by Fick in dividing the rate of oxygen uptake by the arterio-venous oxygen difference to estimate the cardiac blood output. In the work here presented, creatinine and *p*-aminohippuric acid (hereinafter designated as PAH) have been used as the renal excretory substances. The general equation (1) is:

$$1) \quad F_b = \frac{D}{A_b - R_b}$$

The subscript *b* is used to indicate values for whole blood.

F_b = flow of renal blood in liters per minute.

D = excretion rate of excretory substance in milligrams per minute (Am-bard's "debit" (2)).

A_b = concentration of excretory substance in arterial blood, milligrams per liter.

R_b = concentration of excretory substance in renal venous blood, milligrams per liter.

$A_b - R_b$, the whole blood $A - R$ difference, is the amount of excretory substance extracted by the kidneys from 1 liter of renal blood. The fraction, $(A_b - R_b)/A_b$, is the fraction of the arterial content of the substance that is extracted. In accordance with previous usage (3) this fraction will be called the "extraction".

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² The Bureau of Medicine and Surgery does not necessarily undertake to endorse views or opinions which are expressed in this paper.

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The equation is based on five assumptions: (a) that the change in volume which the blood undergoes during its passage through the kidney, as the result of withdrawal of excreted water, is negligible; (b) that the rate of change of arterial blood concentration of the excretory substance during the period of urine collection is so nearly uniform that the concentration at the mid-point of the period can be taken as the mean concentration of the period; (c) that the rate of urine flow is sufficiently rapid to make the excreted volume in the collection period large in comparison with the residue of urine in the collecting tubules and pelvis, so that the urine collected shall represent that formed in the nephrons during the period; (d) that all the specific excretory substance found in the urine comes from the blood (none being formed, like ammonia, in the kidneys). If these conditions are met, equation 1 appears to be valid, regardless of the distribution of the excretory substance between cells and plasma in either arterial or renal venous blood. These conditions have been found to be met for the dog by urea, creatinine, inulin, and ferrocyanide, as excretory substances (1, 3, 4, 5). (e) A fifth assumption is that all the blood that enters the renal vein comes from the kidney. Evidence that such is the case has been presented by Corcoran and Page (23).

b. *Estimation of renal blood flow from excretion rate and plasma A - R difference of an excretory substance.* If a substance meets the above conditions, and in addition is excreted solely by extraction from the *plasma* of the renal blood, none of the substance passing out of the cells during passage of the blood through the kidneys, nor *in vitro* during separation of plasma for analysis, equations 2 and 3, based on plasma analyses, are also valid:

$$2) \quad F_p = \frac{D}{A_p - R_p}$$

$$3) \quad F_b = \frac{D}{(A_p - R_p)V_p}$$

In these equations the symbols have the same significance as in equation 1, except that the subscript, *p*, indicates plasma values, *b*, whole blood values. V_p is the volume of plasma in one volume of whole blood, as determined by hematocrit.

The extra criterion indicating that an excretory substance is valid for application of equations 2 and 3 is that, if any of the substance circulates in the cells, it must show the same concentration in the cells of renal venous blood as in arterial cells. Since extraction of the substance from the plasma by the kidney lowers the concentration of substance in plasma relative to that in the cells, a diffusion gradient from cells to plasma may be established before the blood reaches the renal vein. As a result, significant diffusion of the substance from cells to plasma may occur, either during perfusion of the kidney, or afterwards *in vitro* in the drawn renal venous blood before the plasma can be separated for analysis, unless the rate of diffusion is insignificant. If diffusion occurs while the blood perfuses the nephrons, and some of the diffused substance is excreted,

it will increase D , which for use in equations 2 and 3 must represent substance excreted solely from plasma. If substance diffused from the cells is not excreted, and comes through in the renal venous plasma, or if the diffusion occurs *in vitro* in the drawn renal venous blood, it will increase R_p of equations 2 and 3. From inspection of these equations it is evident that such increases of either D or R_p will cause plus errors in the renal plasma flows calculated.

Inulin (4, 5, 6), mannitol (6), and ferrocyanide (3) meet the requirements of equations 2 and 3 in the dog; they circulate only in the plasma, their concentration in the cells being practically zero. Creatinine also meets the requirements (3, 5); although when injected into the circulation it slowly penetrates the cells, no significant proportion diffuses out of them during perfusion of the kidneys, nor in the time required for rapid centrifugation of plasma from renal blood for analysis (3).

PAH is shown in the present paper to approximate the behavior of creatinine. But in the case of PAH, before centrifugation of the renal venous blood can be finished, enough diffusion occurs from cells to plasma to decrease the PAH difference, $A_p - R_p$, by an approximately constant proportion, viz., 5 per cent. Hence, the true decrease in PAH concentration from arterial to renal venous plasma is 1.05 ($A_p - R'_p$), when R'_p represents the analytically observed PAH concentration in plasma obtained by immediate centrifugation of the drawn renal venous blood. Equations 2 and 3 can therefore be applied with PAH as excretory substance by substituting 1.05 ($A_p - R'_p$) in place of $A_p - R_p$ in these equations (see equation 13).

c. *Estimation of renal blood flow from excretion rate and plasma concentration. Relation of PAH plasma clearance to renal plasma flow.* If an excretory substance in its behavior meets the conditions outlined above under section b, and in addition the fraction E_p of the substance that is extracted from the plasma by the kidneys is constant, one may substitute $E_p A_p$ for $A_p - R_p$ in equations 2 and 3 and thereby simplify them to

$$4) \quad F_p = \frac{D}{E_p A_p} = \frac{\text{plasma clearance}}{E_p}$$

$$5) \quad F_b = \frac{D}{E_p A_p V_p} = \frac{\text{plasma clearance}}{E_p V_p}$$

The ratio, D/A , is the "clearance" defined by Møller, McIntosh, and Van Slyke (7) as the volume of blood (or plasma) that contains the amount of excretory substance that is excreted in one minute; hence, the above definitions of blood flow are in terms of clearance and extraction.

In the case of PAH, data presented in this paper show that the plasma extraction in dogs averages 0.87, with a maximum range of ± 9 per cent from this mean. Hence, within this percentage range of error, one can estimate renal plasma flow in dogs from PAH clearances by substituting, for E_p in equations 4 and 5, the constant, 0.87, or 1/1.15.

$$6) \quad F_p \text{ from PAH} = \frac{1.15D}{A_p} = 1.15 \times \text{plasma PAH clearance.}$$

The observed errors, caused by deviation of plasma PAH extraction from the average 0.87 in a series of plasma flow estimations, are shown by figure 3.

The practical advantage of using equation 4 or 5, as exemplified in equation 6, to estimate renal plasma flow is that the necessary data are obtained without analysis of renal venous blood, and require only the technique of a clearance determination. Renal blood flow can not be thus estimated from the clearances of urea, creatinine, or inulin as excretory substances because their extractions are too inconsistent.

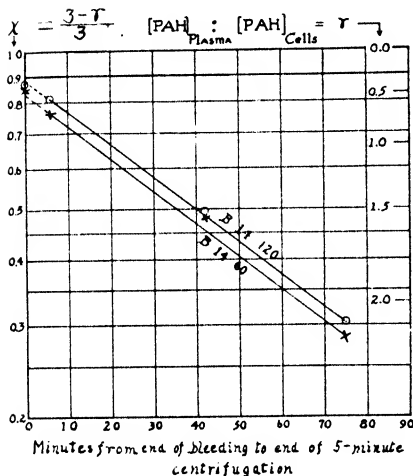


Fig. 1

Fig. 1. Diffusion of para-amino hippuric acid from cells to plasma of renal venous blood *in vitro*. The r scale on the right indicates values of the PAH concentration ratio, plasma:cells; the x scale on the left indicates the fraction of PAH diffused from cells to plasma, if unity represents the total amount required to diffuse to change r from 0 to its equilibrium value, viz., 3. The points for x at the moment the blood was drawn (zero minutes) are calculated on the assumption that the PAH concentration in the cells of renal venous blood at that moment was the same as in the cells of arterial blood. That this assumption is probably correct is indicated by the fact that the extrapolated lines intercept these points.

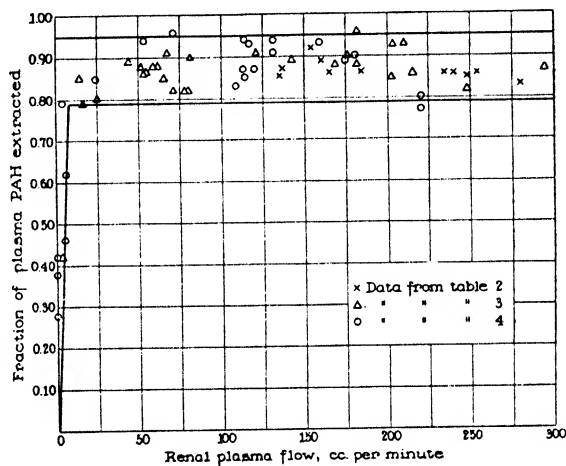


Fig. 2

Fig. 2. Fraction of PAH extracted from the plasma by the kidneys, renal plasma flows being either normal or diminished by hemorrhage or trauma.

White and Heinbecker (8) and Corcoran, Smith, and Page (9) have tested the application of equation 4 with diodrast as excretory substance in dogs and have found respectively mean values of 1.2 and 1.15 for the constant, $1/E_p$.

If an excretory substance could be used which is 100 per cent extracted from the plasma, so that $E_p = 1.00$, equation 4 would simplify to $F_p = D/A_p = \text{plasma clearance}$. Diodrast was introduced by Smith, Goldring, and Chasis (10) as a substance which approximated this requirement in man: its plasma clearances were found to approach so nearly the probable renal plasma flows that it was evident that extraction approached completeness, and that diodrast

clearances could be taken as at least approximate minimal values of renal flow. Later, Smith (11) and his collaborators introduced PAH as a substitute for diodrast; they found PAH to have the same plasma clearance as diodrast, and to have the practical advantage of simpler analytical determination. Warren, Brannon, and Merrill (12) have recently devised a technique for catheterizing the human renal vein, and found that the extraction of PAH from the renal

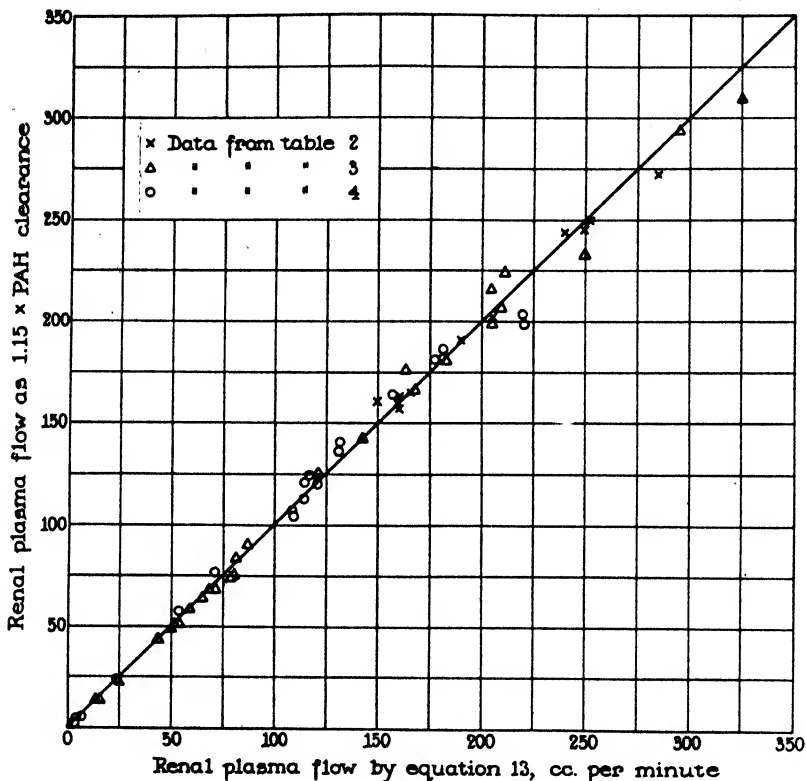


Fig. 3. Constancy of ratio between the para-amino hippuric acid clearance and the renal plasma flow calculated from extraction and excretion by equation 13.

plasma averaged 88 per cent complete, practically identical with the 87 per cent found in our dogs.

d. *Estimation of glomerular filtration rate.* Contributions from the laboratories of A. N. Richards, H. W. Smith, J. A. Shannon, and from this laboratory, previously reviewed (3), indicate that inulin, creatinine, ferrocyanide, and mannitol (6) are excreted by the dog,⁴ purely by the process of glomerular fil-

⁴ In the case of man only inulin and mannitol appear to possess all of these properties. Ferrocyanide is, like urea, about 40 per cent reabsorbed in the human tubules (Miller and

tration, and that the plasma clearances of these substances may be taken as equivalent to the volume of filtrate filtered in the glomeruli per minute. In the present studies the creatinine clearance has been used, chiefly because of the relative convenience of the technique for determination of creatinine (13).

e. *Estimation of fraction of plasma water filtered in the glomeruli.* Van Slyke, Hiller and Miller (3) have presented evidence that the fraction, E_p , of creatinine, inulin, or ferrocyanide, extracted from the plasma during perfusion of the dog's kidneys, is equal to the fraction of the plasma water, usually 0.2 to 0.3, that is filtered in the glomerulus.⁵

$$7) \quad E_p(\text{Cr}) = \frac{A_p - R_p}{A_p} = \text{filtered fraction of plasma water}$$

where A_p and R_p indicate concentrations of creatinine in the plasma of the arterial and renal venous blood, respectively, and (Cr) indicates creatinine.

EXPERIMENTAL. *Operative procedure.* Female dogs varying in weight from 11 kgm. to 23 kgm. were used. Anesthesia was induced by nembutal in an initial dose of 29 mgm./kgm., given intravenously as a 6.5 per cent solution in 10 per cent alcohol solution and was maintained by intravenous infusion at an average rate of about 0.04 mgm./kgm./min. Plasma concentrations of creatinine and hippurate were established by an initial intravenous injection of about 70 mgm./kgm. and 10 mgm./kgm. respectively, and were maintained by intravenous infusion of these substances in saline at a rate of 10–15 mgm./min. and 4–6 mgm./min., respectively, until the beginning of hemorrhage or trauma, when these rates were halved by dilution of the infusion fluid to prevent excessive rise of plasma concentration. The infusion rate of nembutal was then also halved.

In the experiments of table 1 and table 2 the animals were not sacrificed, and dogs were used in which the left renal vein had been made accessible by transplanting the kidney to a position under the skin of the flank. The operative technique followed was that of Rhoads (16), with the modification that the renal vein was dissected free from the ureter and the renal artery, and that the separated fibers of the external oblique muscle were sutured together between the renal vein and the rest of the renal pedicle; the renal vein was thus placed outside the layer of muscle separating the vein from the renal artery and ureter. This location prevents accidental insertion of a needle into the renal artery or ureter when blood is being drawn from the renal vein. After healing, the right kidney was removed. These operative preparations were finished six months or more before the dogs were used for the experiments of tables 1 and 2.

In the experiments of tables 3 and 4, in which severe shock was induced, arterial blood pressures were followed and the animal was sacrificed at the end of

and Winkler, 14); and creatinine, when its plasma concentration is raised by administration or renal retention, appears to be partly excreted by the human tubules, because its plasma clearance is then greater than the inulin clearance (15).

⁵ In the present work the extracted fraction of plasma creatinine, E_p (Cr), has been used as a measure of the filtered fraction of plasma water.

TABLE 1
Diffusion of para-amino hippurate from cells to plasma *in vivo* and *in vitro*

EXPERIMENT NO.	TIME		ARTERIAL BLOOD						RENAL VENOUS BLOOD						PLASMA EXTRACTION		
	Start of PAH infusion to bleeding	Bleeding to centrifuging	Cell volume V_c	PAH concentrations				Cell volume V_c	PAH concentrations				Uncorrected $\frac{E_p - R_p}{A_p}$	E_p corrected for diffusion from cells by use of K_p from Eq. 12	E_p uncorrected per cent of E_p corrected		
				Plasma A_p	Cells A_c	Whole blood A_b			Plasma R_p	Cells R_c	Whole blood R_b						
						By direct analysis	Calc. as $\frac{V_c A_c + V_p A_p}{V_p A_p}$				By direct analysis	Calc. as $\frac{V_c R_c + V_p R_p}{V_p R_p}$					
																mg./l.	mg./l.
B 1	5	0	0.351	85.2	16.0	60.9	0.19	0.376	105.2	83.5	95.4	97.0	0.51	0.53	96		
Oct. 12, 1942		20		81.8	18.3	59.5	0.22		112.6	70.4		96.8	0.48	0.53	90		
		60		81.0	21.3	60.1	0.26		114.8	63.9		95.6	0.47	0.54	87		
	10	0	0.375	68.0	16.8	48.8	0.25	0.396	229.7	156.0	200.8	200.3	0.36	0.38	95		
B 10		20		67.5	17.7	49.4	0.26		245.0	134.7		201.4	0.31	0.38	82		
		40		67.5	19.4	50.1	0.28										
	120	0	0.394	357	173.2	284.4	0.50	0.435	30.0	42.2	34.5	35.4	0.78	0.82	95		
B 14		0	0.433	132.7	50.8	97.0	0.38	0.435	40.3	25.6		33.9	0.70	0.84	84		
		36							45.0	20.0		34.0	0.66	0.84	79		
	80																
Oct. 22, 1942		0	0.442	128.7	49.6	93.7	0.39	0.442	23.2	41.6	31.5	31.3	0.82	0.87	94		
		36							38.5	25.6		32.8	0.70	0.85	82		
	80								43.0	19.2		32.5	0.67	0.85	79		

each experiment; dogs with explanted kidneys were not used. Renal venous blood was obtained in each experiment by exposing a renal vein. After induction of anesthesia, a carotid cannula was inserted and connected with a mercury manometer. An inlying catheter was introduced into the bladder and fixed in place with adhesive strapping. This was followed by laparotomy and exposure of the left renal vein. The right kidney remained undisturbed. It was assumed that the renal blood flows and extractions observed were representative of both kidneys, and were not seriously affected by anesthesia and laparotomy—assumptions which find support in the correspondence of renal blood flow and

TABLE 2

Comparison of renal plasma flows estimated simultaneously with creatinine and para-amino hippuric acid

Also data on glomerular filtration in dogs without shock

EXPERIMENT	CREATININE						PARA-AMINO HIPPIURIC ACID						Ratio $\frac{F_p (Cr)}{F_p (PAH)}$	
	Plasma concentrations		Excretion rate D	Plasma flow $\frac{1000 D}{F_p = \frac{A_p - R_p}{A_p}}$	Clearance $C_{Cr} = \frac{1000 D}{A_p}$ (glomerular filtrate)	Extraction $E_p = \frac{A_p - R_p}{A_p}$ (filtered fraction)	Plasma concentrations		Excretion rate D	Plasma flow $\frac{95 D}{F_p = \frac{A_p - R_p}{A_p}}$	Clearance $C_{Cr} = \frac{1000 D}{A_p}$	Extraction $E_p = \frac{1.05 (A_p - R_p)}{A_p}$		
	Arterial A_p	Renal venous R_p					Arterial A_p	Renal venous R_p (uncorrected)						
B 18 Oct. 27, 1942	168.8 189.0 207.0 216.5	124.5 139.2 152.5 164.0	11.31 12.55 13.74 15.38	257 257 252 293	67.0 66.4 66.4 71.1	0.263 263 263 247	33.0 36.8 39.4 40.1	6.0 6.7 7.4 8.3	7.34 8.04 8.38 9.50	240 254 249 284	216 218 213 237	0.86 86 85 83	1.07 1.01 1.01 1.03	
B 19 Oct. 29, 1942	207.0 241.0 273.2 292.0	156.0 181.0 204.0 220.0	7.72 9.56 11.28 11.90	151 159 163 165	37.3 39.7 41.3 40.7	0.246 252 253 247	55.0 63.5 72.2 75.4	6.8 9.7 12.4 14.3	7.56 9.04 9.90 10.18	152 160 157 159	140 142 137 135	0.92 89 87 85	0.99 0.99 1.03 1.02	
B 20 Nov. 4, 1942	126.8 130.5 136.5	97.8 100.5 104.5	4.78 7.28 6.24	165 243 195	37.7 55.8 45.0	0.229 230 234	16.2 17.3 18.1	2.9 3.1 3.3	2.32 3.50 2.93	165 235 188	143 202 162	0.86 86 86	1.00 1.03 1.04	
Average.....													0.865	1.02

creatinine extraction data observed in these dogs with data from unanesthetized dogs with explanted kidneys (1, 5). Because of the bladder relaxation induced by the anesthesia, it was important to recover all urine by washing the bladder with saline at each collection period. Urine was received from the inlying catheter into a 100 cc. volumetric flask; then the bladder was rinsed twice with 20 cc. portions of saline and emptied by manual compression, and the total volume of urine plus washings was made to 100 cc.

After laparotomy two or three clearance periods of about 30 minutes were run with sampling of arterial and renal venous blood at approximately the mid-point of each period. If the two samples were not drawn simultaneously, the

TABLE 3

Creatinine and para-amino hippuric acid excretion in hemorrhagic shock

DOG NO. WEIGHT, SURFACE AREA	TOTAL BLOOD LOSS	BLOOD PRESSURE	CREATININE					PARA-AMINO HIPURIC ACID					RATIO $\frac{F_p}{F_p} \left(\frac{Cr}{PAH} \right)$		
			Plasma concentrations		Excretion rate D	Plasma flow $F_p =$ $\frac{1000 D}{A_p - R_p}$	Clearance $C_{Cr} =$ $\frac{1000 D}{A_p}$ (glomerular filtrate)	Extraction $E_p =$ $\frac{R_p}{A_p - R_p}$ (fraction)	Plasma concentrations		Excretion rate D	Plasma flow $F_p =$ $\frac{1000 D}{A_p - R_p}$		Clearance $C_{Cr} =$ $\frac{1000 D}{A_p}$	Extraction $E_p =$ $\frac{R_p}{1.05(A_p - R_p)}$ Eq. 14
			Arterial A_p	Renal venous R_p					Arterial A_p	Renal venous R_p uncor- rected					
	cc.	mm.	mg./l.	mg./l.	mg./min.	cc./min.	Eq. 2	Eq. 7	mg./l.	mg./l.	mg./min.	cc./min.	Eq. 13†	Eq. 14	
B 21 Feb. 15, 1943 22.6 kg.	40 80 730 910 950 990 1190 1290	130 125 125 130 120 110 80 80	168.2 168.5 135.6 135.6 139.2 139.2 270.0 304.0	124.5 129.0 90.0 89.0 89.4 88.0 319.4 325.5	13.32 13.37 9.24 9.24 8.46 8.00 0.45 0.40	305 339 203 200 170 156	79 79 68 69 61 60 1.7 0.1	0.260 0.234 0.334 0.343 0.358 0.368 (-0.184) (-0.070)	22.6 21.7 15.6 16.8 18.4 18.6 50.6 84.6	3.8 4.7 1.8 2.0 2.6 1.6 56.6 68.6	5.81 5.81 3.04 3.16 2.93 2.86 0.08 0.02	294 326 210 203 176 162 1.6 0.2	257 268 195 188 159 154	0.87 0.82 0.93 0.93 0.90 0.96 (-0.13) 0.21	
0.96 m ²															
B 22 Feb. 19, 1943 17.2 kg.	60 500 540 580 660 700 740 780 820 860 900 940	140 105 110 108 106 98 85 85 95 85 75 60	182.8 141.0 132.8 125.8 124.3 122.8 129.5 145.0 167.2 194.5 221.3 251.0	143.0 106.4 98.2 92.0 93.2 94.0 98.8 117.7 146.7 173.5 201.8 263.5	10.87 7.60 6.94 6.19 5.61 4.77 4.42 2.41 2.96 2.21 1.10 0.08	273 220 201 183 180 166 144 88 144 105 56	60 54 52 49 45 39 34 17 18 11 5 0	0.218 0.245 0.260 0.260 0.250 0.234 0.237 0.188 0.123 0.108 0.088 (-0.050)	22.6 12.4 11.7 11.4 11.6 11.7 13.4 16.1 19.8 26.0 32.6 43.4	5.0 2.2 2.2 1.9 1.9 1.8 1.8 2.2 2.8 4.8 7.7 26.2	4.58 2.29 2.03 1.81 1.69 1.47 1.47 61 81 53 25 3	248 214 204 182 166 142 110 61 73 45 20 1	0.82 0.86 0.85 0.88 1.00 1.06 1.17 1.19 1.31 1.78 1.98 2.24		
0.76 m ²															
B 23 March 15, 1943 21.3 kg.	640 680 720 760 800 840 880 880 920 960 1000 1040	80 85 90 94 86 88 88 88 85 80 55 50	373 388 395 398 404 413 421 421 428 482 488 499	325 337 325 312 328 344 339 334 333 402 402 449	2.88 3.36 4.52 4.78 4.26 4.17 5.24 5.08 4.08 1.30 1.06	60 66 65 59 56 61 64 54 41 20 21	7.7 8.7 11.4 12.0 10.5 10.1 12.5 11.9 9.4 2.8 2.1	0.129 0.132 0.177 0.202 0.189 0.166 0.195 0.219 0.230 0.141 0.100	51.6 55.0 55.8 55.2 56.3 57.4 56.7 60.4 69.3 78.5	11.5 12.2 12.2 10.4 10.2 9.0 9.0 9.0 12.9 18.7	3.28 3.18 3.55 3.01 2.55 3.38 2.88 2.30 0.86 1.04	78 71 78 64 55 53 67 58 43 14 16	64 58 58 55 45 45 59 51 38 12 13	0.77 0.93 0.84 0.92 1.06 1.19 0.95 0.93 0.98 1.42 1.31	
0.90 m ²															

Time relations are shown in figures 6, 7 and 8.

† The change from the constant 0.95 of equation 13 in the text to 950 here is due to the fact that the values of A_p and R_p in this table are in terms of liters, while the plasma flow, for convenience, is in terms of cc.

TABLE 4
Creatinine and para-aminohippuric acid excretion in traumatic shock

DOG NO.	L = LAPAROTOMY M = MUSCLE TRAUMA	BLOOD PRESSURE	CREATININE					PARA-AMINO HIPURIC ACID					RATIO $\frac{F_p (Cr)}{F_p (PAH)}$		
			Plasma concentration		Excretion rate $\frac{D}{A_p - R_p}$ Eq. 2	Plasma flow $\frac{F_p}{1000 D}$ $\frac{A_p - R_p}{A_p}$ Eq. 2	Clearance $\frac{C_p}{1000 D}$ (glomerular filtrate) Eq. 6	Extraction $\frac{R_p}{A_p - R_p}$ (filtered fraction) Eq. 7	Plasma concentration		Excretion rate $\frac{D}{A_p - R_p}$ Eq. 13	Plasma flow $\frac{F_p}{1000 D}$ $\frac{A_p - R_p}{A_p}$ Eq. 13		Clearance $\frac{C_p}{1000 D}$ $\frac{A_p - R_p}{A_p}$ Eq. 14	Extraction $\frac{R_p}{1.05 (A_p - R_p)}$ Eq. 14
			Arterial A_p	Renal venous R_p					Arterial A_p	Renal venous R_p					
T 1 Feb. 23, 1943	L M	mm.	mg./l.	mg./l.	mg./min.	cc./min.	mg./min.	mg./l.	mg./l.	mg./min.	cc./min.	cc./min.	mg./min.	cc./min.	
		110	281	223	13.83	236	49	0.207	24.0	3.4	3.91	181	163	0.90	
		90	290	229	14.21	233	49	0.210	22.0	5.2	3.89	220	177	0.80	
		78	321	271	8.19	161	25	0.158	20.4	3.6	2.13	121	104	0.87	
		80	325	263	7.56	122	23	0.190	20.8	3.6	2.05	113	99	0.87	
	M	60	524		0.66	1		7.6		0.02		0.3			
T 2 Feb. 26, 1943	L M	96	163	119	7.20	163	49	0.270	10.6	2.8	1.81	221	171	0.77	
		90	114	86	5.08	184	45	0.242	5.2	0.89	0.89		171		
		105	237	167	8.77	125	37	0.295	26.3	5.0	2.53	113	96	0.85	
		110	259	184	8.76	116	34	0.290	28.2	6.0	2.53	108	90	0.83	
		88	431	423	0.07	9	0.2	0.020	62.0	37.0	0.02	0.7	0.3	0.42	
T 3 March 18, 1943	L	100	507	491	0.11	6	0.2	0.033	89.8	56.5	0.03	0.9	0.4	0.39	
		85	594	532	0.07	1	0.1	0.096	182.8	133.5	0.03	0.6	0.4	0.28	
		130	223	158	11.24	173	50	0.291	22.5	3.4	3.55	177	158	0.89	
		140	229	156	11.73	161	51	0.319	24.9	2.8	3.65	157	147	0.93	
		130	239	157	8.59	104	36	0.344	27.6	3.0	2.91	113	105	0.94	
	M	135	252	158	12.22	131	48	0.371	30.8	3.2	3.83	132	124	0.94	
		135	257	164	11.39	122	44	0.363	32.6	3.6	3.56	117	109	0.93	
		140	259	168	12.52	136	48	0.354	32.6	4.4	3.89	131	119	0.91	
		110	304	206	6.33	65	21	0.321	41.0	4.3	2.03	53	50	0.94	
		110	346	243	7.56	73	22	0.297	49.0	4.1	3.35	71	68	0.96	
	M	85	405	326	2.95	38	7	0.194	66.1	12.4	1.38	24	21	0.85	
		80	458	401	0.56	10	1.2	0.125	81.3	20.0	0.29	4	4	0.79	
		80	504	470	0.53	2	1.0	0.068	99.3	40.5	0.41	7	4	0.62	
		70	568	553	0.31	2	0.5	0.027	128.2	72.2	0.29	5	2	0.46	

The successive blood and urine samples were drawn at intervals of about 30 minutes.

arterial plasma concentrations of PAH and creatinine at the moment the renal venous blood was drawn were estimated by graphic interpolation on time curves of the arterial concentrations.

The preliminary periods completed, experimental shock was induced either by hemorrhage or trauma.

Hemorrhagic shock was induced by an initial rapid drawing of 200–500 cc. of blood. Further bleedings were carried out as found necessary to maintain the blood pressure below 70–80 mm. of mercury and to produce a further gradually progressive fall. The total quantity of blood removed, including samples for analysis, amounted to 3 to 5 per cent of the body weight.

Traumatic shock was induced by the method of Gregersen (17) slightly modified. The animal under nembutal anesthesia received an initial trauma, distributed over both thighs, of about 50 blows per kilogram body weight with a 2-pound rawhide mallet. More blows were administered as necessary to maintain the blood pressure depression and to induce a further slowly progressive fall.

Observation of clearance and extraction values continued during both types of shock over the subsequent two to six hours, usually until shock had progressed to suppression of glomerular filtration, which occurred variably at blood pressures between 40 and 100 mm.

ANALYTICAL METHODS. *Blood samples* were drawn at approximately the midpoint of each urine collection period; a sample was first drawn from the femoral artery, then one from the renal vein. To minimize diffusion of PAH between cells and plasma in the drawn blood, the latter was centrifuged in the cold as quickly as possible. Samples of 15 to 20 cc. were drawn through no. 19 needles into chilled oiled syringes, and were transferred quickly to 15 × 100 mm. "lusteroid" tubes each containing 1 mgm. of heparin. The tubes were capped, inverted twice, then spun at once in an angle centrifuge kept at 4°. Centrifugation continued at 4500 RPM for 5 minutes. With the exception of those experiments of table 1 in which the effect of delay in centrifugation was tested, less than a minute elapsed between the finish of drawing the blood and the beginning of centrifugation.

p-Aminohippuric acid. Following Finkelstein, Aliminosa, and Smith (11), PAH in blood and urine was determined by applying the diazo method developed by Bratton and Marshall (18) for determination of free sulfonamides in blood filtrates. For the present analyses plasma filtrates were prepared by diluting 2 cc. of plasma to 30 cc. with water and adding 10 cc. of 15 per cent trichloroacetic acid. Whole blood or red cells were prepared by diluting 2 cc. to 20 cc. with water and adding 20 cc. of the 15 per cent trichloroacetic acid. The color was developed usually in 10 cc. portions of filtrate. If the concentration of PAH exceeded 6 mgm./100 cc. of plasma, however, a portion of 5 cc. or less of filtrate was taken and diluted to 10 cc. after the addition of 1 cc. of 1.2 N HCl to insure acidity adequate for diazotization.

For urine analyses from 1 to 8 cc. of the 100 cc. portion of urine plus washings was acidified by the addition of 2 cc. of 1.2 N HCl and then further diluted to 10 cc., after which it was treated like the blood filtrates.

Measurement of the optical density was made in a spectrophotometer at a wave length of 530 millimicrons. The zero point of the photometer was set with distilled water. Correction for the optical density due to normal blood constituents was made from blank values obtained with samples of plasma or blood that had been drawn before the injection of PAH. These were treated by the same procedure as the subsequent samples. It is essential, in both the unknowns and blanks, that the readings be taken 15 minutes after addition of the naphthyl-ethylenediamine reagent; otherwise, turbidity and non-specific color may develop.

Complete recoveries of added PAH were obtained in analyses of plasma and urine, but the amounts found in the filtrates from whole blood and cells were only 94 and 92 per cent, respectively, of the amounts added—the remainders apparently having been lost by adsorption on the protein precipitates. Accordingly all PAH concentrations obtained for whole blood and red cells have been divided by 0.94 and 0.92, respectively, to correct for this loss.

Creatinine was determined by the Folin-Wu method as modified by Phillips (13).

Hematocrit readings were made in Wintrobe tubes which had been spun 45 minutes at 3000 RPM in a centrifuge with an 18 cm. radius to the center of the tube. In experiments to estimate the amount of plasma trapped in the cell column, the dye T-1824 was added to the blood before centrifugation. The trapped plasma was calculated under the assumptions, discussed by Gregersen and Schiro (19), that no dye penetrates or is absorbed by the cells, and that the dye found in the column is dissolved in the trapped plasma. It was thus estimated that 4.5 per cent of the volume of the cell column consisted of plasma. In estimating the reported V_c values in table 2 from the hematocrit readings, the latter were accordingly multiplied by 0.955.

RESULTS AND DISCUSSION. I. *Validity of PAH extraction and excretion values, and of PAH clearance, for calculation of renal plasma flow in normal and shocked dogs.* 1. *Effects of PAH diffusion between cells and plasma in vivo and in vitro.* a. *Diffusion between cells and plasma in the general circulation.* In the experiments summarized in table 1 samples of renal arterial and venous blood were taken at intervals after the start of continuous infusion of PAH. A portion of each blood sample was centrifuged immediately to separate the cells from the plasma, while other portions were allowed to stand at room temperature for different intervals, as indicated in the table, before centrifugation. While the bloods were standing, they were stirred by inversion every 5 minutes to keep the cells in suspension.

The data in table 1 on PAH concentrations in arterial cells show that when PAH is injected into the circulation it diffuses into the blood cells, the diffusion behavior resembling that previously noted for creatinine (3). It appears that in the general circulation equilibrium between cells and plasma with regard to PAH concentration is approached in somewhat less than an hour.

Equilibrium appeared to be approached in the circulating blood when the concentration of PAH in the cells was about 0.4 as great as in the plasma (arterial ratio, $A_c : A_p = 0.4$). Thus, in experiment B 14, where the PAH con-

centration was kept nearly constant during the period from 60 to 120 minutes after the start of PAH infusion, the $A_c : A_p$ ratio changed during this period only from 0.38 to 0.39. The ratio 0.40 approximates the distribution ratio of diffusible monovalent anions between cells and plasma according to the Gibbs-Donnan theory (20), when concentrations are calculated as moles per liter of cells and plasma.

b. *Diffusion of PAH between cells and plasma in vitro.* When renal venous blood from PAH-infused dogs was permitted to stand, a fairly rapid diffusion of PAH from cells to plasma occurred, evidenced by decrease in the successive cell R'_c values and increase in the plasma R'_p .⁶ In the arterial blood (B 10) such diffusion was relatively slight. The progress of the diffusion is shown by the decrease in the $R'_c : R'_p$ ratio in successively centrifuged samples of the same renal venous blood. The obvious reason for the diffusion from the renal venous cells *in vitro* was that the kidneys had extracted the greater part of the PAH from the plasma, and relatively little from the cells, of the renal blood, thereby creating a steep diffusion gradient of PAH from cells to plasma in the blood emerging from the kidneys. That the progressive fall in the ratio was entirely due to diffusion, and not to destruction of PAH in the cells, is shown by the fact that the PAH content of the whole blood remained unchanged during the standing *in vitro*.

c. *The improbability that significant proportions of PAH leave the blood cells during perfusion of the kidneys.* Comparison of the A_c and R'_c values of experiments B 10 and B 14 in table 1 shows that even the PAH concentrations found in the cells of renal venous blood subjected to the quickest possible centrifugation after drawing (R'_c values for zero "bleeding to centrifuging" time) were lower than the PAH concentrations found in the cells of simultaneously drawn arterial blood. The question arises, whether this apparent decrease in cell PAH from arterial to renal venous blood occurred during post-nephron perfusion of the kidneys, or whether it was an artifact due to diffusion of PAH from cells to plasma *in vitro* during the few minutes required to draw and centrifuge the renal venous blood.

In order to answer this question we have used a function of the plasma:cell PAH concentration ratio which shows a linear change with diffusion during the time that the PAH-charged cells stand in contact with the relatively PAH-depleted plasma. Extrapolation of the linear time curve back to the moment when the renal venous blood was drawn indicates that the speed of diffusion *in vitro* was of the order of magnitude to cause the observed $A_c - R'_c$ differences, and hence that it is not necessary to assume the occurrence of extraction of cell PAH by the kidneys to explain these differences.

To obtain the linear function it was assumed that the amount of PAH diffusing per minute from cells to plasma at any time, t , is proportional to the amount

⁶ R_c and R_p indicate the concentrations of PAH found in the cells and plasma of renal venous blood centrifuged after standing for the indicated intervals, while R_c and R_p indicate the concentrations in the renal venous blood at the moment it was drawn.

that must still diffuse before equilibrium is reached. As a measure of this amount we have taken x , defined as:

$$8) \quad x = \frac{a - r}{a},$$

where r represents the plasma:cell concentration ratio of PAH at any time, t , and a represents the value of r when equilibrium is reached. If t_0 is the initial moment when $r = 0$, then at any time, t , x is the fraction of the total diffusion (the total occurring from t_0 until attainment of equilibrium) that still remains to be achieved after time, t . For diffusion equilibrium *in vitro*, a was found to approximate 3, rather than the value, 2.5 (viz. 1/0.4) that appeared to be approximated *in vivo*.⁷

The velocity assumption made is expressed as

$$9) \quad \frac{dx}{dt} = -kx.$$

The equivalent form,

$$10) \quad \frac{d \log x}{dt} = -k,$$

indicates the linear relation between $\log x$ and t .

In figure 1 are plotted the data obtained from the two renal venous blood samples drawn in experiment B 14. Each time period from the moment the blood was drawn is estimated by adding to the "bleeding to centrifuging" interval in table 1, 6 minutes for the time required to draw and centrifuge the blood. It is obvious that diffusion speed during these 6 minutes would not be exactly the same as the subsequent diffusion speed when the blood was standing in the tubes; during the drawing and the beginning of centrifugation diffusion would presumably be faster, while during the latter part of centrifugation, when separation of cells approached completion, it would be slower. As a rough approximation, however, it is assumed that the two factors balanced, and that the mean diffusion rate during the 6 minutes required to draw and centrifuge the blood was approximately the same as though the blood had been standing.

The value of x at the moment the blood was drawn (zero minutes in fig. 1) was calculated by assuming that no loss of PAH from the cells occurred in the kidneys, and hence that $R_c = A_c$. The value of R_p at this moment was calculated on the same assumption by equation 12.

The fact that in figure 1 the linear curves extrapolated to the moment the bloods were drawn approximately intercept the x values estimated as above for the freshly drawn blood, indicates that the rate of diffusion of PAH from cells to plasma in drawn renal venous blood *in vitro* was such that such diffusion

⁷ Equations 8, 9, and 10 are approximate expressions for the diffusion rate which serve the purpose of linear extrapolations and are simpler than the equations derivable from Fick's law for a system such as plasma and cells. Equations 8, 9, and 10 do not cover the condition that $a < r$, which is never reached when the initial excess of PAH is in the cells.

during the few minutes taken to draw, transfer, and centrifuge the blood, could account for the observed $A_e - R'_e$ difference in cell PAH.

Graphic estimation of the k value of equations 9 and 10 indicates that at any moment the rate of diffusion per minute was of the order of magnitude of 1 per cent of the PAH, diffusion of which was still required to produce equilibrium.

The improbability of the diffusion of significant proportions of PAH from the cells during perfusion of the kidneys is made evident by a consideration of the brevity of time available for such diffusion. The mean volume of blood flowing through the kidneys of normal dogs has been found to be about 4 cc. per minute per gram of kidney (1). Hence a dog with kidneys weighing 80 grams would have a renal blood flow of about 320 cc. per minute. One may estimate the amount of blood in the kidney at a given moment at a maximum of 10 per cent of the weight of the organ (21). The 80 grams of kidneys would thus be estimated to contain 8 cc. of blood. With a blood flow of 320 cc. per minute, the mean time required for a particle of blood to flow through the kidney would therefore be $\frac{8}{320}$ or $\frac{1}{40}$ of a minute. Of this interval, only a part would be spent in the period after the blood reaches the tubules. It is only in that period that the diffusion gradient of PAH from cells to plasma is established, as the result of tubular excretion of unfiltered plasma PAH, and as the result of plasma dilution by reabsorption of water. In the four analyses of experiments B 10 and B 14 in which R'_e was determined in cells obtained by immediate centrifugation, R'_e was from 0.84 to 0.91 of A_e . For 9 to 16 per cent of cell PAH to have diffused to plasma during perfusion of the kidney would require diffusion to be 400 to 600 times as fast *in vivo* as *in vitro*. Data are not available to calculate the velocity constant of equations 9 and 10 *in vivo*, but in experiment B 1, table 1, the ratio of cell PAH to plasma PAH increased in 5 minutes only from 0.19 to 0.25, indicating that the diffusion *in vivo* does not have enough speed to produce a significant fall in R_e in 1.5 seconds.⁸

e. *Correction of PAH extractions, and of renal blood flows calculated from them, for in vitro diffusion of PAH from cells to plasma in renal venous blood.* From the preceding discussion it appears that the observed fall of the PAH concentration

⁸ In studies of the excretory behavior of diodrast in dogs, White and Heinbecker (8) found, as we have for PAH, that the diodrast concentration in cells centrifuged from the renal venous blood was lower than in cells from arterial blood. These authors also calculated the renal plasma flow in two ways, (a) with inulin as excretory substance by equation 2, and (b) with diodrast as excretory substance, whole blood flow was calculated by equation 3, and was multiplied by V_p to estimate plasma flow. The plasma flow from diodrast by procedure *b* averaged 1.12 times the flow from inulin by procedure *a*. Both the higher flow value thus obtained with diodrast, and the apparent decrease in cell diodrast concentration occurring as the blood perfused the kidney, could be explained either by excretion of diodrast from the cells into the urine, with increase of the D of the equation, or by *in vitro* diffusion of diodrast from cells to plasma in the drawn venous blood, with resultant decrease in the difference, $A_p - R'_p$. White and Heinbecker preferred the first of these explanations, excretion of cell diodrast, because they considered that diffusion of diodrast between cells and plasma *in vitro* was too slow to be significant in the time required to centrifugalize the renal venous blood. If this explanation is correct, diodrast must diffuse more rapidly than PAH *in vivo* and more slowly *in vitro*.

in renal venous cells, centrifuged immediately after the blood was drawn, below the concentration in arterial cells is practically all attributable to diffusion from cells to plasma *in vitro* during the interval required for the drawing and centrifuging of the renal venous blood. The diffusion producing the observed decrease, $A_c - R'_c$, in PAH concentration from the cells of arterial to those of renal venous blood must produce an increase in the PAH concentration of the renal venous plasma, the changes in the cell and plasma PAH concentrations being inversely proportional to the cell and plasma volumes, respectively. Hence:

$$11) \quad (A_c - R'_c):(R'_p - R_p) = V_p:V_c, \text{ and}$$

$$12) \quad R_p = R'_p - \frac{V_c(A_c - R'_c)}{V_p}$$

The calculation of the corrected value, R_p , appears to be accurate because the " E_p corrected" values, calculated by equation 7, from R_p values calculated by equation 12 in the next to the last column of table 1, remain constant, within the limit of analytical accuracy, in successively centrifuged portions of each renal venous blood, while the uncorrected E_p values decrease markedly in the successively centrifuged samples.

Those results in the last line of table 1 from blood centrifuged immediately after drawing indicate that in renal venous blood thus centrifuged the uncorrected value of the difference $A_p - R'_p$, obtained by PAH determinations on the separated plasma, was 4 to 6 per cent below the true difference, and that when the renal venous blood was allowed to stand before centrifugation was begun, the error was increased, being about 15 per cent if the blood stood 40 minutes. Introducing a 5 per cent correction into equations 2 and 3 yields equation 13, which is valid for PAH values if the R'_p values are those yielded by PAH analyses of plasma from renal venous blood centrifuged immediately after it is drawn.

$$13) \quad F_p(\text{PAH}) = \frac{D}{1.05(A_p - R'_p)} = \frac{0.95 D}{A_p - R'_p}$$

Similarly, with the 5 per cent correction, the extraction of PAH from plasma is calculated by equation 14:

$$14) \quad E_p(\text{PAH}) = \frac{1.05(A_p - R'_p)}{A_p}$$

The correction must increase with the V_c of the blood, but with V_c varying, in table 1, from 0.38 to 0.44, the correction factor increased only from 1.04 to 1.06.

It will be noted in table 1 that dog B 10 showed unusually low PAH extracted fractions, 0.53 and 0.38 (corrected), compared with values usually in the range 0.84–0.90 from the other dogs, shocked and unshocked, in tables 1, 2, and 4. The low plasma extractions in B 10 are attributable to tubular saturation by the high arterial plasma PAH concentration in this animal. As shown by Smith and Shannon and their collaborators (5, 10), for substances eliminated, like PAH,

by tubular excretion, when the plasma concentration passes a certain limit excretion ceases to rise in proportion to plasma concentration, because the tubular part of the mechanism for excreting the substance approaches a saturation limit beyond which it cannot be accelerated, and tubular excretion rate approaches the " T_m " limit.

2. *Comparison of renal plasma flows calculated from extraction and excretion of creatinine and of PAH, respectively, by equations 2 and 13.* Van Slyke, Hiller, and Miller (3) have shown that the immediately centrifuged cells of renal venous blood from normal dogs injected with creatinine contain the same concentration of creatinine as cells from simultaneously drawn arterial blood. Hence measurement of plasma creatinine extraction appears, at least in normal dogs, to suffer no significant error from diffusion from cells to plasma in the renal venous blood during either perfusion of the kidney or immediate centrifugation, and plasma flows calculated from creatinine values by equation 2 should be free from error due to diffusion.

The results with unshocked dogs, in the last column of table 2, show that renal plasma flows calculated by equation 13 from PAH plasma values average within 2 per cent of renal plasma flows calculated by equation 2 from creatinine values. The agreement is within the limit of experimental error.

With progressing shock (last lines of each experiment in tables 3 and 4) the agreement between plasma flows estimated with creatinine and PAH, respectively, becomes less close, but except in B 22 remains within the limit of experimental error until the flows fall to 3 or 4 per cent of normal. Then the entire function of the kidney becomes anomalous; thus, in the last periods of B 21 and B 22 the creatinine concentration in the renal venous plasma was found greater than in the arterial; "extraction" became negative. Either the kidneys were forming and passing into the blood some other substance that gave the color reaction of creatinine, or creatinine stored in the kidneys was diffusing back into the blood, the flow rate of which had slowed to almost zero.

It appears that the flows estimated from PAH values are more reliable in shock than the flows estimated from creatinine. Even with normal renal function the extraction of creatinine is only about $\frac{1}{4}$ that of PAH, so that in the simultaneously determined values of A_p and R_p errors of 2 per cent in opposite directions could make an error of about 20 per cent in flow values estimated by creatinine (equation 2) and only about 5 per cent in flows estimated by PAH. Also, it is known that non-creatinine substances giving the color reaction for creatinine exist in the blood, chiefly in the cells, but entering the plasma under abnormal conditions (22). It is possible that these substances significantly affect the values of plasma flow estimates based on creatinine in severe shock. Because the effect of analytical error is less in estimating the PAH-estimated flows, and because the blood does not appear to contain constituents of its own which interfere with PAH determination, it appears that in shocked dogs renal plasma flows estimated from PAH values are less subject to error than those estimated from creatinine values, despite the fact that a 5 per cent correction to the PAH-estimated flow is necessary for PAH diffusion from cells to plasma *in vitro*.

3. *The accuracy with which renal plasma flows can be estimated from PAH clearances (equation 6) in shocked and unshocked dogs.* The results obtained in 3 dogs without shock and in 6 with varying degrees of hemorrhagic and traumatic shock are summarized in tables 2, 3, and 4. The plasma PAH extraction (corrected for diffusion from cells to plasma during centrifugation) covers the same range in all three tables, if the values from observations during shock so severe that renal plasma flow was below 7 cc. per minute (about 3 per cent of normal) are excluded (see fig. 2). With exclusion of these values, the mean PAH extraction is 0.87 with a standard deviation of ± 0.04 .

From the fact that the extraction, $(A_p - R_p)/A_p$, is approximately constant at 0.87 for PAH, it follows that one may substitute $0.87 A_p$, or $A_p/1.15$, for $A_p - R_p$ in equation 2 to calculate approximate values of F_p . This substitution leads to equation 6.

The range of variation in the PAH extraction, estimated either as twice the standard deviation, or graphically from figure 2, is approximately ± 9 per cent of the mean in all observations where the renal plasma flow exceeded 7 cc. per minute (about 3 per cent of normal). Hence, except in cases with less than 3 per cent of normal renal blood flow, in renal plasma and blood flows calculated by equation 6, a maximum error of ± 9 per cent can be expected, due to deviations of the actual extraction from the assumed 0.87. The actual errors from this source, in plasma flows calculated as $1.15 \times$ PAH clearance, are indicated in figure 3 by the vertical distances of the experimental points from the 45-degree line.

When the renal blood flow was depressed by shock to 2 or 3 per cent of normal, the proportion of PAH extracted from the plasma diminished (fig. 2) and the renal plasma flow estimated as 1.15 times PAH clearance developed a plus error which was significant in percentage of the slight flow measured, but was slight in absolute magnitude, because the total flow was so small (see fig. 3).

Corcoran and Page (23) have studied the extraction and excretion of diodrast and inulin by the kidney during hemorrhages in dogs such that blood pressure was lowered to 60 mm. Such reduction of blood pressure frequently lowers renal function to the range of 2 to 3 per cent of normal discussed in the preceding paragraph. Our results (Figs. 6, 7, 8) confirm the finding of Corcoran and Page that with shock of such severity the filtered fraction (creatinine extraction in our experiments, inulin extraction in Corcoran and Page's) is depressed, so that the rate of glomerular filtration is relatively retarded even more than the renal blood flow. Also our finding that when renal function is depressed to this range the fraction of plasma PAH excreted falls (Fig 2), so that PAH clearance decreases relatively even more than renal blood flow, is similar to observations made by Corcoran and Page with diodrast in place of PAH. Their observations do not include the intermediate stages of hypotension (blood pressure over 70 mm.) where we found PAH extraction maintained at 0.80–0.95 (Fig. 2) and the filtered fraction sometimes even increased above usual levels.

II. EFFECTS OF HEMORRHAGIC AND TRAUMATIC SHOCK ON RENAL PLASMA FLOW, FILTRATION, AND EXCRETION. From the data in tables 3 and 4 and in

figures 4-8 the following conclusions may be drawn concerning the course of events relating renal function and circulatory changes in hemorrhagic shock in the dog:

Sudden massive hemorrhage causes an immediate drop of blood pressure, which, if the fall is below a level which may vary from 60 to 100 mm., is accompanied by almost complete cessation of renal blood flow and function as measured by PAH clearance (fig. 4). The approximately complete stoppage of renal blood flow indicates presumable constriction of the renal arterioles. If the blood loss is not too great, the systemic arterial pressure soon rises, presumably because of extra-renal peripheral constriction, which seems, in sudden

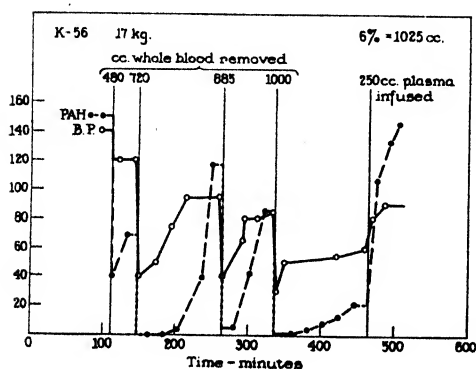


Fig. 4

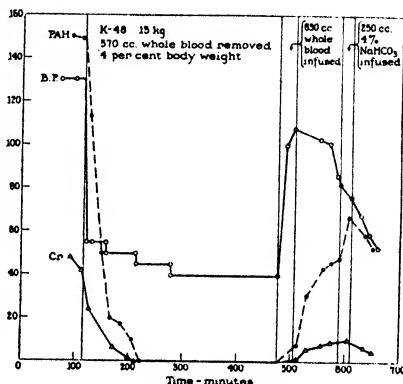


Fig. 5

Fig. 4. Repeated rapid hemorrhages, each followed by peripheral vasoconstriction, with recovery of blood pressure and renal function. Favorable reaction near end of experiment to infusion of volume of plasma smaller than volume of blood withdrawn. Solid circles indicate PAH plasma clearance in cc. per minute, hollow circles indicate blood pressure in mm. of mercury. Figures for whole blood removed are total summated volumes.

Fig. 5. Effects of severe hemorrhage. Replacement of lost blood delayed so long that complete replacement caused only temporary restoration of blood pressure and renal function. PAH indicates PAH plasma clearance in cc. per minute, Cr., creatinine plasma clearance, B.P., blood pressure in mm. of mercury.

hemorrhage, to occur somewhat more slowly than renal constriction. When peripheral constriction has raised blood pressure to a level which varies between 60 and 100 mm., the circulation to the kidneys is resumed, although at less than pre-hemorrhage flow rate (fig. 4). The kidneys appear now to be favored at the expense of the other peripheral circulation. (That complete pre-shock circulation could not well be restored to the kidneys after massive hemorrhage can be deduced from the fact that ordinarily the kidneys take about one-fourth of the resting blood flow).

The cycle of sudden hemorrhage, drop in blood pressure, shut-down of the kidneys, and subsequent partial restoration of blood pressure and renal function can be repeated two or more times in the same animal, until the limit of tolerated

blood loss has been reached (fig. 4). Even then, restoration of blood pressure and partial renal function may be accomplished, at least temporarily, by injection of a volume of blood or plasma only a fraction of that which has been lost (fig. 4). The reason for this effect is apparently that peripheral constriction reduces the volume of the vascular bed to such an extent that replacement of only a part of the lost blood suffices to raise the blood pressure and to restore renal circulation.

If the state of blood depletion persists too long (fig. 5) replacement even of all the lost blood causes only a temporary rise in blood pressure and in renal blood flow and excretion. Peripheral constriction is replaced by peripheral dilatation, and renal function soon falls again.

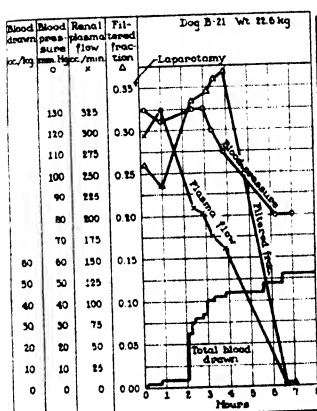


Fig. 6

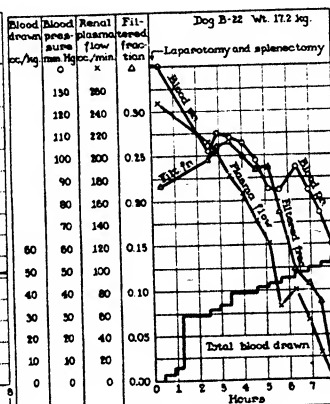


Fig. 7

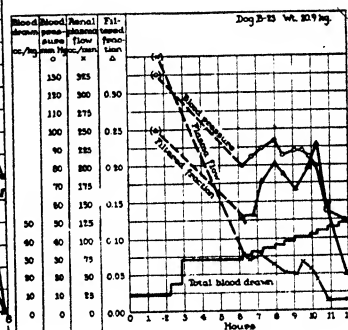


Fig. 8

Fig. 6. Effects of progressive hemorrhage.

Fig. 7. Effects of progressive hemorrhage.

Fig. 8. Effects of progressive hemorrhage.

The fraction of plasma water filtered in the glomeruli, as measured by the creatinine extraction, does not decrease *pari passu* with falling plasma flow, nor with the systemic blood pressure. In fact, the filtered fraction may increase as shown in figures 6, 7 and 8, while the renal plasma flow is falling. Such maintenance, and even increase in, the filtered fraction appears to indicate constriction of the efferent glomerular arterioles to such an extent that filtration pressure in the glomeruli is maintained, and even increased, despite falling general arterial pressure, and despite decreased blood flow through the kidney.

Eventually with progressing shock a stage is reached in which there is failure of the compensatory efforts of peripheral constriction to maintain a functioning level of blood flow to the kidneys, and a failure of efferent glomerular constriction to maintain filtration. There is then a rapid fall both of renal blood flow and of the fraction of plasma water filtered in the glomeruli (later stages, figs. 6, 7 and 8).

The onset of complete renal failure depends upon the *duration* of the state

of blood deficit as well as upon the volume of blood lost. Thus, in figure 6 complete renal failure is seen developing during a 2-hour period in a dog that had for a time established a fair degree of compensation for his blood loss, and was not further bled, except for blood samples taken for analysis.

The stage of complete, or nearly complete, failure of renal function is reached when the general blood pressure is still at a level of 60 to 100 mm. It appears that at this stage constriction of the afferent renal arterioles shuts off the renal circulation, sacrificing the renal blood flow in an apparent effort to maintain circulation to vital centers. The cessation of excretion does not appear to be due entirely to lack of sufficient arterial pressure to maintain glomerular filtration; in dog T 2 (table 4) practically complete cessation of excretion occurred with a general blood pressure of 100 mm., whereas in T 1 a renal plasma flow and PAH clearance of about half normal value were maintained with a general arterial pressure of only 78-80 mm.

The effects of muscle trauma (table 4) appear to be similar to those of hemorrhage, except that after trauma there was a failure to note the transitory drop of blood pressure and cessation of renal function, followed by recovery, that was observed after sudden limited hemorrhage. When blood pressure fell after trauma there appeared to be less tendency for it to rise again. Otherwise, relations between the general blood pressure, renal blood flow, and glomerular filtration appear to be essentially the same as in hemorrhagic shock.

Reversibility of renal damage in dog and man. In the dog, as exemplified in figures 4 and 5, renal function damaged by acute hemorrhagic shock appears to be capable of restoration until the general peripheral vasoconstriction has given way to vasodilatation, and the animal can no longer maintain blood pressure, even when it is temporarily restored by transfusion. Man appears to differ from the dog in that after severe and prolonged shock with anuria, it may be possible, in a man in shock, to restore the general circulation by transfusion, without restoring enough renal function to maintain life, which is terminated after some days by uremia. The ratio of resistance of the kidney to resistance of the rest of the organism, with regard to the effects of prolonged shock, appears to be greater in the dog than in man. As will be shown in a later paper, the dog's kidneys do have a limit of resistance; if complete renal ischemia is maintained in the dog for 2 to 4 hours by clamping the renal arteries, the kidneys suffer irreversible renal damage and subsequent uremic death results, similar to that observed in man after restoration from prolonged severe shock with anuria. But either the kidneys of the dog are relatively more resistant to the ischemia produced by shock, or extra-renal factors involved in recovery from shock are less resistant in the dog, for attempts to prolong hemorrhagic shock in the dog long enough, or to produce traumatic shock severe enough to cause subsequent uremia in the dog have regularly resulted in death without recovery from the acute shock, before there was time for uremia to develop.

SUMMARY

Extraction of *p*-aminohippuric acid from the plasma of blood perfusing the kidneys of dogs has been found to be 87 per cent complete, with a standard

deviation of ± 4 per cent. In estimating the extraction, a correction was found necessary for diffusion of *p*-aminohippuric acid from cells to plasma in the renal venous blood during the drawing and centrifugation of the blood; by use of rapid and uniform technique this correction could be kept down to 5 ± 1 per cent. Acute hemorrhagic or traumatic shock did not alter the percentage extracted, unless shock was so severe that the renal blood flow was retarded to less than about 3 per cent of normal. Except in this extreme condition, the renal plasma flow could be calculated as the plasma *p*-aminohippuric acid clearance/0.87, with an error within the range ± 9 per cent, and usually less than 5 per cent.

After sudden hemorrhage of 20 to 30 cc. per kilo the sequence of events observed in dogs under nembutal anesthesia was a drop in arterial blood pressure to 50–60 mm., with cessation of measurable renal blood flow and excretion, followed quickly by partial or nearly complete restoration of central blood pressure and of renal function. The restoration appeared to be attributable to constriction of the extra-renal peripheral vessels, and to indicate that renal circulation was favored at the expense of extra-renal peripheral circulation.

However, if further progressive hemorrhage followed, over the approximate range, 30 to 40 cc. per kilo., this blood loss was accompanied by decrease in renal blood flow, although central blood pressure might be maintained above 110 mm. During this period the fraction of plasma water filtered in the glomeruli, as measured by the creatinine extraction, tended to increase, and thereby to uphold the volume flow of glomerular filtrate despite the shrinkage in renal blood flow. The circulatory phenomena during this period apparently included partial constriction of the afferent renal vessels, adding renal to peripheral constriction in the endeavor to maintain central blood pressure, while the efferent renal vessels constricted still more than the afferent, to produce the compensatory increase in the fraction of plasma water filtered.

When hemorrhage surpassed a certain limit, about 40–45 cc. per kilo, or when blood lost by somewhat smaller hemorrhage was not replaced for some hours, or when muscle trauma exceeded a certain limit, both renal blood flow and the fraction of plasma water filtered fell to almost zero levels. This renal debacle might occur when the central blood pressure was still 80–100 mm. It appeared that by maximum afferent renal constriction the organism at this stage temporarily strangled renal function in an effort to maintain central blood pressure. If not too severe or prolonged, the condition in this stage was still reversible by infusion of blood or plasma, or by spontaneous recovery.

The relations between central blood pressure, renal blood flow, and glomerular filtration in shock caused by muscle trauma were similar to the relations noted in shock caused by hemorrhage.

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THE RENAL EXTRACTION OF OXYGEN IN EXPERIMENTAL SHOCK^{1, 2}

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Anoxia appears to be an important consequence of shock, different tissues being affected to different degrees. One sign of this anoxia in general is increased extraction of oxygen from the blood,⁴ apparently in partial compensation for the reduced volume flow of blood through the tissue. This phenomenon is a matter of familiar clinical experience in the peripheral circulation where darkening of the venous blood occurs concomitantly with reduced volume flow. A study of the renal oxygen extraction in shock was, therefore, undertaken in order to evaluate the possible rôle of anoxia as a cause of damage to the kidney. Contrary to expectation it was found that the renal extraction of oxygen in early shock is but moderately increased, despite a marked reduction in the volume flow of blood.

Under normal conditions, as shown originally by Claude Bernard (1), the kidneys extract a smaller proportion of oxygen from the blood perfusing them than is extracted by most other tissues. This observation has been confirmed in experiments with dogs (2) and with human subjects (3, 4). As compared with the removal of 4 to 6 volumes per cent of oxygen by the body as a whole under basal conditions, the kidneys remove only 1.5 to 3.5 volumes per cent.

EXPERIMENTAL AND ANALYTICAL PROCEDURES. Hemorrhagic shock was produced and maintained in dogs under nembutal anesthesia, and the renal veins were exposed, as previously described (5). As in those experiments (5), the renal blood flow was calculated from the excretion rates and extractions of both p-aminohippuric acid and creatinine. The flow values, thus independently measured, usually checked each other within about 3 per cent. The kidneys were removed and weighed at the end of each experiment.

In addition to the blood samples taken for renal blood flow estimations, further samples were taken of femoral arterial, renal venous and, occasionally, right auricular blood (by catheter passed in the right jugular vein). The blood was received directly into tonometers over mercury (6) for determination of the oxygen content by the method of Van Slyke and Plazin (7), using 1 cc. samples.

¹ The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Hospital of The Rockefeller Institute for Medical Research.

² The Bureau of Medicine and Surgery does not necessarily undertake to endorse views or opinions which are expressed in this paper.

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⁴ See equation 7.

The blood was kept in a bath of ice water while awaiting analysis—an interval of less than three hours.

For measurement of the capacity of the blood to bind oxygen as HbO_2 , portions of approximately 3 cc. were brought to room temperature and saturated with air in rotating 50 cc. tubes. During the period of saturation, about 20 minutes, the air contained in each tube was changed three times.

The amount of oxygen bound by hemoglobin as HbO_2 per liter of blood was estimated by subtracting, from the total blood O_2 , a correction for the physically dissolved O_2 (see equations 1, 2 and 3, below). The physically dissolved O_2 was calculated from the solubility coefficients found by Sendroy, Dillon and Van Slyke ((8) and p. 326 of (7)) for O_2 in blood, and from assumed O_2 tensions of 155 for blood saturated with air at 23° (for O_2 capacity), 100 mm. at 38° for arterial blood (from data on dogs by Drabkin and Schmidt) (9), and 50 mm. at 38° for renal venous blood. The physically dissolved O_2 thus calculated was 6.5 cc. per liter for blood saturated with air at 23° , 3.0 cc. for arterial blood, and 1.4 cc. for renal venous blood. It is estimated that such deviations from the assumed O_2 tensions as may have occurred would not affect results sufficiently to alter significantly the calculated O_2 consumption of the kidney.

It was found, in accord with Van Slyke, Rhoads, Hiller and Alving (2) that there was usually a significant difference in O_2 capacity (hemoglobin concentration) between renal venous blood and femoral arterial blood drawn within a few minutes of each other (table 1).⁵ Technique was not available for drawing serial samples from the renal artery. Therefore the concentration of oxygen entering the kidney in the renal arterial blood was estimated (equation 5) under the two assumptions that the renal arterial blood had the same hemoglobin content (i.e., oxygen capacity) as that found in the emergent renal venous blood, while the per cent saturation of its hemoglobin was the same as that of the femoral arterial blood.

⁵ The cause of the frequent difference in hemoglobin concentration between bloods from the femoral artery and the renal vein is still uncertain. One conceivable cause may be a minute-to-minute variation in the hemoglobin content of the general circulation, which might be marked in dogs because of the splenic reservoir of erythrocytes. Another possibility might be a difference in cell content between aortic arterial blood and renal arterial blood, due to hydrodynamic effects at the point where the renal artery branches off. The difference does not appear to be attributable to concentrating effect of water excretion by the kidneys, because the volume of water excreted per minute is usually of the order of only $\frac{1}{10}$ or less of the volume of blood perfusing the kidney; also if water withdrawal were the cause, the renal venous blood would regularly show higher Hb concentration than blood from the femoral artery, whereas the differences are about as often in the opposite direction (table 1). Nor does the difference appear to be attributable to hemodilution immediately following withdrawal of the first sample of blood (about 20 cc.) of each pair; the samples were of the order of only one or two per cent of the circulating blood, and the interval between the two samples of each pair was too short for inflow of extra-circulatory fluid to be appreciable; in table 1 it is seen that the [Hb] of the second sample is sometimes greater, sometimes less, than the first. The assumption made, that the blood during passage through the kidney does not significantly change its hemoglobin concentration, however, seems justified.

In the following equations the subscripts FA, RA, RV refer to femoral artery, renal artery and renal vein, respectively. Let:

[Hb] = active hemoglobin content of blood measured by oxygen binding capacity in cc. O₂ per liter. (This does not include methemoglobin or carbon monoxide hemoglobin.)

[O₂] = total oxygen content of blood (cc. O₂ per liter)

[HbO₂] = oxygen bound by hemoglobin (cc. O₂ per liter)

S = percentage oxygen saturation of hemoglobin

F = renal blood flow (liters per minute)

Q = rate of oxygen consumption by the kidneys (cc. per minute)

The preceding definitions are expressed by the equations:

$$1) [\text{Hb}] = [\text{O}_2] - 6.5 \quad (\text{aerated blood})$$

$$2) [\text{HbO}_2]_{\text{FA or RA}} = [\text{O}_2]_{\text{FA or RA}} - 3.0 \quad (\text{arterial blood})$$

$$3) [\text{HbO}_2]_{\text{RV}} = [\text{O}_2]_{\text{RV}} - 1.4 \quad (\text{renal venous blood})$$

$$4) S = \frac{[\text{HbO}_2]}{[\text{Hb}]} \times 100$$

$$5) [\text{O}_2]_{\text{RA}} = [\text{Hb}]_{\text{RV}} \times \frac{S_{\text{FA}}}{100} + 3.0$$

$$6) Q = F ([\text{O}_2]_{\text{RA}} - [\text{O}_2]_{\text{RV}})$$

$$7) \text{Oxygen extraction} = \frac{[\text{O}_2]_{\text{RA}} - [\text{O}_2]_{\text{RV}}}{[\text{O}_2]_{\text{RA}}}$$

RESULTS AND DISCUSSION. Oxygen extraction by the kidney increased only slightly with early shock, despite marked decrease in renal blood flow. This failure of the kidney to increase its oxygen extraction contrasted with the marked increase in oxygen extraction by the body as a whole (table 1, fig. 1). The contrast was evident from visual inspection of the blood samples. Before shock the arterial, renal venous, and right auricular blood presented the bright color of well oxygenated blood, becoming somewhat darker in the order given. By analysis they were about 95 per cent, 85 per cent and 75 per cent saturated with oxygen respectively. While the arterial and renal venous samples in early or moderately advanced shock showed no appreciable change in appearance, blood obtained at the same time from the right auricle or from a peripheral vein was darkened to a marked degree. It was only when shock became advanced to such a degree that renal blood flow was reduced to a few cubic centimeters per minute, as judged from the outflow from a transected renal vein, that the renal venous blood became dark. The darkening of the renal venous blood which then occurred was due to increased renal extraction of oxygen, not to incomplete arterial oxygenation. The important generalization brought out by the present experiments is that when renal blood flow decreases in shock renal oxygen consumption shows a proportional or almost proportional decrease.

Experiments were performed to find which of several possible explanations might account for the failure of the kidney to increase its oxygen extraction from the blood when the renal blood flow decreased.

C	112 (initial hemor- rhage) 92	{ 123 134 196 }	211.7 —	— 222.7 (204.1)	— 194.2 185.2	90.3 —	— 82.6	— 18.9	— 9.3	— 5.40	— 102	
		{ 250 256 }	190.1 —	— 167.9 (133.7)	— 151.0 128.6	77.9 —	— 75.8	— 5.1	— 3.8	— 1.86	— 9	
D	168	{ 10 28 }	205.7 —	— 216.8 (193.7)	— 183.9 180.6	88.0 —	— 82.7	— 13.1	— 6.8	— 6.13	— 80	
	(hemor- rhages) 74	100-160 { 246 256 }	— 182.4	— 180.5 (154.5)	— 92.5 156.1	— 84.0	— 50.4	62.0 —	40.0 —	0.17 —	11 —	
E	164	{ 42 53 }	216.0 —	— 221.8 (203.9)	— 198.6 179.1	90.6 —	— 80.1	— 24.8	— 12.2	— 6.58	— 163	
	(hemor- rhages) 90	110-145 { 235 240 }	203.6 —	— 221.9 (197.5)	— 181.4 153.7	87.6 —	— 68.6	— 43.8	— 22.2	(too low to meas- ure)	— —	
		262	—	—	—	—	—	—	—	—	201.5 31.6 15	
F	138	{ 23 39 }	225.5 —	— 224.0 (207.9)	— 209.3 183.8	91.5 —	— 81.4	— 24.1	— 11.6	— 6.72	— 162	
	(hemor- rhages) 190	85-135 { 243 257 }	240.2 —	— 243.1 (224.8)	— 222.1 209.8	91.2 —	— 85.7	— 15.0	— 6.8	— 3.84	— 58	
	120	{ 273 285 }	— —	— 243.3 (224.9)	— 210.1	— —	— 85.8	14.8	6.6	3.84	57	
	120		—			—					241.5 64.1 26	

* Figures in parenthesis are values of renal arterial oxygen content $[O_2]_{RA}$ estimated from equation 5, from $[Hb]_{AV}$ and the S_{PA} of the femoral arterial blood drawn just before or after the renal venous.

One such explanation was that the kidney might be unable to extract oxygen from the blood when the oxygen tension in the capillaries of the kidney fell below a certain level. An experiment (table 2) to test this hypothesis indicated that it was untenable. A dog was made to breathe a mixture of 8 per cent oxygen in

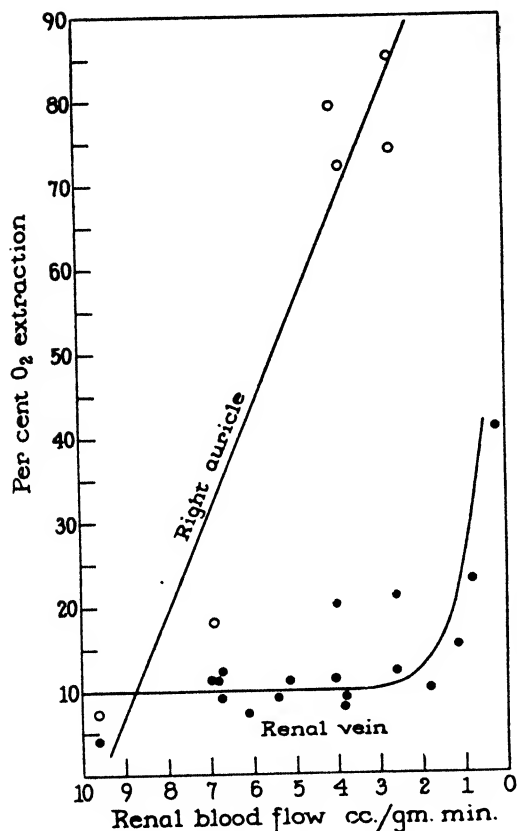


Fig. 1

Fig. 1. Comparison of the renal oxygen extraction (●), and the oxygen extraction by the body as a whole (○), computed from the oxygen contents of arterial and right auricular blood, both related to the renal blood flow in seven hemorrhagic shock experiments.

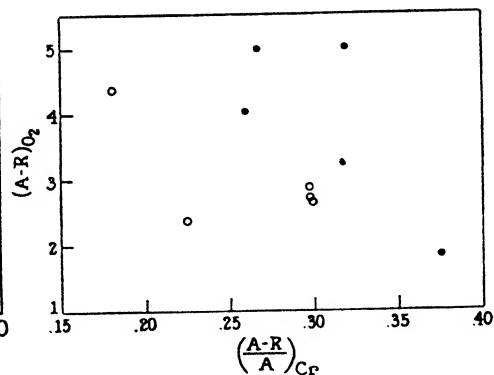


Fig. 2

Fig. 2. Lack of correlation between the renal arteriovenous oxygen difference, $(A - R)O_2$, and creatinine extraction $\left(\frac{A - R}{A}\right)_{Cr}$ in two experiments with adrenalin infusion.

nitrogen, thereby lowering the arterial oxygen saturation to the minimum tolerated by the nervous system. Despite this reduction in arterial oxygen tension the kidneys continued to consume oxygen at about the same rate as in the control period. In so doing the kidneys lowered the renal venous oxygen

saturation to unusually low levels. It is therefore evident that the failure of the kidney to increase oxygen extraction with retardation of the blood flow is not due to inability to lower the renal venous oxygen tension below its ordinary level.

A second possibility considered was that blocks of kidney tissue might be excluded from the circulation in such a way that the velocity of blood flow in the remaining regions would be normal despite reduction in volume flow through the organ as a whole. In three experiments dogs under nembutal anesthesia were prepared by laparotomy with exposure of both renal pedicles, and then brought into hemorrhagic shock by the technique used in oxygen extraction experiments. Shortly after the blood pressure had been brought below 70 mm. of mercury, an injection of 5 cc. of dye (India ink, Evans blue or trypan blue) was made into a renal artery, followed by clamping of the renal pedicle 30 seconds after completion of the injection. The kidney was immediately removed and sectioned for gross examination. This procedure was repeated with the remaining

TABLE 2

Failure of decrease in oxygen of inspired air to decrease renal oxygen consumption

INSPIRED GAS	ARTERIAL BLOOD		RENAL VENOUS BLOOD		RENAL OXYGEN REMOVAL		RENAL BLOOD FLOW	RENAL OXYGEN CONSUMPTION
	Oxygen content	Oxygen saturation	Oxygen content	Oxygen saturation	Arterio-venous difference	Per cent extraction		
	cc./liter	per cent	cc./liter	per cent	cc./liter	per cent	liters/min.	cc./min.
O ₂ , 21 per cent N ₂ , 79 per cent (atmosphere).....	205.0	96	174.4	82	30.6	15	0.245	7.5
O ₂ , 8 per cent N ₂ , 92 per cent.....	145.3	63	120.5	52	24.8	17	0.379	9.4

kidney. In all cases the staining was quite uniform throughout the parenchyma, except for occasional unstained regions less than 3 mm. in diameter. The second hypothesis, therefore, appeared to be untenable.

Thirdly, it was considered that the energy requirement of the kidneys might be dependent on glomerular filtration rate, since this is normally proportional to the rate of oxygen consumption—renal blood flow being proportional to both. To test such a possibility the usual fixed relation between renal blood flow and glomerular filtration (i.e., the filtration fraction) was varied by the intravenous infusion of adrenalin at a rate of 0.03 to 0.04 mgm./min. Under these conditions the hypothesis would predict a correlation between oxygen consumption and filtration rate. For purposes of the experiment it was more convenient to look for the equivalent correlation between the renal arteriovenous oxygen difference and the filtration fraction (which was measured by creatinine extraction (10)). No such correlation was found when the creatinine extraction was varied from 0.18 to 0.38 in two experiments (fig. 2). It was therefore concluded

that the renal oxygen requirement was not determined by the glomerular filtration rate.

Thus the problem of accounting for the failure of the kidneys to increase their oxygen extraction in early shock, despite an ability to do so at a later stage, remains unsolved. It should not be inferred from the sharp decline in oxygen consumption in shock that the kidneys necessarily suffer from anoxia under these conditions, since no direct evidence is available to allow an estimation of the rate of renal oxygen supply that is required to prevent development of either reversible or irreversible damage to the kidney.

SUMMARY

1. The normally small renal arterio-venous oxygen difference remained small in early hemorrhagic shock, despite marked decrease in renal blood flow.

2. As a result, the rate of oxygen consumption by the kidneys decreased markedly even in early shock, almost in proportion to the decrease in renal blood flow.

3. The failure of the kidney in shock to attempt to maintain its oxygen consumption by increasing its oxygen extraction contrasted with the behavior of the rest of the body, which responded with a great increase in its oxygen extraction, as found by oxygen determinations on blood from the right auricle.

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THE EFFECT OF OXYGEN PRESSURE ON THE UPTAKE OF CARBON MONOXIDE BY MAN AT SEA LEVEL AND AT ALTITUDE¹

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Wherever the internal combustion engine is used the potential hazard of carbon monoxide intoxication must be anticipated; the contamination of air with CO from engine exhaust and other sources may occur to varying degrees in all sorts of sea, land and aircraft. Frequently, the specific function for which the craft was designed prevents the absolute exclusion of CO from contact with men inside the craft. There arises, then, the necessity for specifying the permissible limits for contamination of inspired air with CO. There are several factors which must be considered in arriving at such a specification; one of these factors is the rate at which man will take up CO. There are recorded a series of investigations of this factor; the results of these former studies and some of the striking discrepancies have been summarised recently by Forbes, Sargent and Roughton (1). All of these former studies were executed at sea level where the effect of increased or diminished partial pressure of oxygen (pO_2) in the inspired air was not studied. With respect to aircraft the effect of diminished pO_2 assumes great importance; for the anoxia induced by the absorption of CO can only sum with the anoxia produced by flight at altitude and thus add to the already formidable array of physiological stresses to which the aviator is exposed.

This present communication is one of a series of studies from this laboratory dealing with the general problem of CO intoxication in aviation. The rate of uptake of CO in man has been studied at sea level and under conditions which increased or decreased the pO_2 of inspired air. Many of the factors studied have paralleled the concurrent studies at the Fatigue Laboratory, Harvard University, which have been reported recently (1).

METHODS. The subjects studied in these experiments were 8 men and one woman. In all, 78 separate observations were made; 66 of the observations were limited to 4 of the male subjects. Body weights varied from 55 to 95 kgm. and heights from 165 to 185 cm. Body surface areas varied from 1.60 to 2.19 sq. m.

All uptake studies were made with the subject recumbent or sitting quietly so that minute respiratory volumes varied from 6 to 12 l./min. The duration of exposure to the CO mixture varied from 20 to 66 minutes. Subjects were not exposed to CO more than once in a 24 hour period.

The desired gas mixtures were made up in a Tissot spirometer and transferred to a thick rubber bag of 1000 liters capacity. Analyses of mixtures stored in this

¹ The opinions and assertions contained herein are the private ones of the writers and do not reflect the official policies of the Navy Department or the naval service at large.

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bag showed that no loss of CO occurred during the course of an experiment. The mixtures were composed of appropriate amounts of pure CO, air and when required, aviator's breathing oxygen (99.5 per cent O_2). The range of inspired CO tensions (pCO) studied was 0.37 to 2.30 mm. Hg and the per cent concentration of CO varied from 0.05 to 0.275. The concentration of CO in the gas mixtures was checked by analyses with the MSA instrument and by a colorimetric technic. The pO_2 range was 97 to 770 mm. Hg and the percentage of O_2 ranged from 20.9 to 99.5 per cent. The gas mixtures were delivered through check valves and the subject breathed through a mouth-piece with nose-clip.

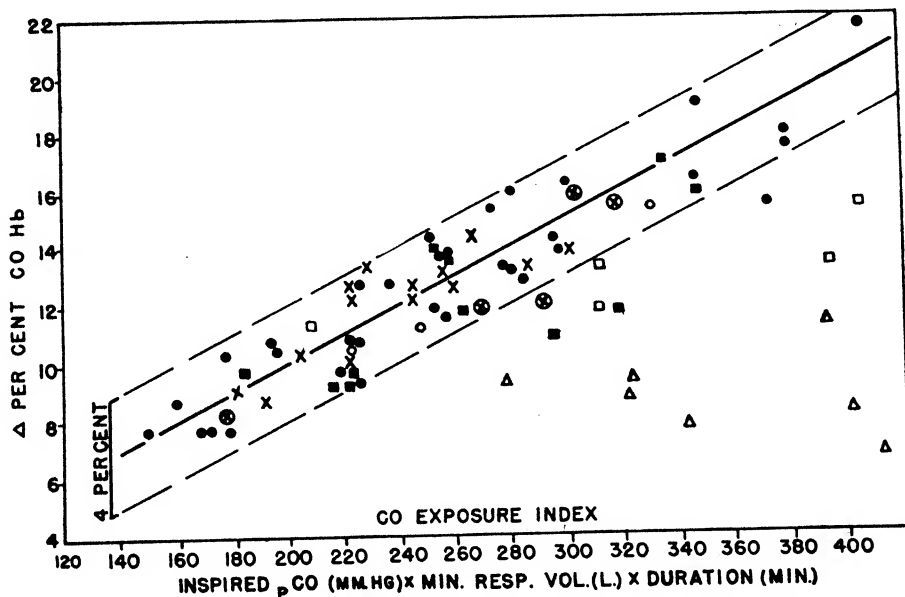


Fig. 1. The rate of uptake of CO by man. ●—Sea level ($pO_2 = 160$ mm. Hg); ○—Sea level ($pO_2 = 300-450$ mm. Hg) 20 min.; □—Sea level ($pO_2 = 300-450$ mm. Hg) 60 min.; ■—Sea level ($pO_2 = 760-770$ mm. Hg) 02 min.; △—Sea level ($pO_2 = 760-770$ mm. Hg) 60 min.; ×—7,000-13,000 feet ($pO_2 = 97-123$ mm. Hg); ⊗—15,000-25,000 feet ($pO_2 = 150-160$ mm. Hg).

Reduction in pO_2 and in total barometric pressure (P_b) was produced in a decompression chamber evacuated to produce the required simulated pressure-altitude. Altitudes from 7,000 to 13,000 were employed to study CO uptake while breathing air; at altitudes from 15,000 to 25,000 the breathing mixtures were enriched with added O_2 to produce a pO_2 equivalent to sea level conditions. The effect of increased pO_2 was studied at sea level by enriching the inspired gas mixture with O_2 .

The CO content of blood was estimated by the microgasometric method of Scholander and Roughton (6). Samples of cutaneous blood were obtained from the finger tip without milking. The CO content of the blood was determined

before exposure to CO because several of the subjects were smokers and their blood contained significant quantities of CO (up to 1.5 vols. per cent). The amount of CO taken up was calculated as the difference between the amounts determined before and after exposure and expressed as the percentage of hemoglobin combined with CO [Δ (COHb)]. A constant total hemoglobin capacity of 19.5 vols. per cent was assumed in each case.

RESULTS. The results of these studies are presented in figure 1. In order to compare rates of CO uptake under a variety of conditions, certain of the significant variables have been combined in a "CO exposure index". This index is the product of the following variables: pCO of the inspired mixture (in millimeters Hg), the minute respiratory volume (in liters) and the duration of exposure (in minutes). The uptake at sea level was found to be linear (solid circles); and this linear relationship may be simply expressed:

$$\Delta(\text{COHb}) = (\text{pCO} \times t \times \text{m.r.v.}) 0.05$$

where pCO = partial pressure of CO in mm. Hg

t = duration of exposure in minutes and

m.r.v. = minute respiratory volume in liters expressed at ambient temperature and water saturation but at a constant pressure of 760 mm. Hg.

Of the 34 observations at sea level, 33 deviated 2 per cent or less from the curve (solid circles). Since all these experiments were carried out at rest the minor variations in circulatory rate have been ignored. Likewise the volume of circulating blood has been ignored; the linearity of the experimental data suggests that the ratio of blood volume to respiratory minute volume remained relatively constant from individual to individual so that blood volume may be omitted from the expression.

Eighteen of the 19 observations of CO uptake at simulated pressure-altitudes of 7,000 to 13,000 feet while breathing ambient air and at 15,000 to 25,000 feet while breathing air-oxygen mixtures equivalent to sea level fell within the same limits as those observed at sea level (crosses and circled crosses).

The effect of increased pO_2 (300–450 mm. Hg) at sea level is indicated in the figure by open circles (20 min. exposure) and open squares (60 min. exposure). The effect of greater pO_2 (760–770 mm. Hg) is indicated by solid squares (20 min.) and triangles (60 min.). The effect of increased pO_2 is reflected in the reduced uptake of CO for a given CO exposure index as compared with the uptake at a pO_2 of 160 mm. Hg or less. The effect, however, is irregular, and 10 of the 25 experiments show no difference from the anticipated uptake at normal pO_2 . It may be noted that the greater deviations occur in the longer exposures.

The total barometric pressure in these experiments ranged from 770 to 282 mm. Hg. This range of variation in P_b produced no demonstrable effect on the rate of uptake of CO.

The subjective symptoms accompanying the absorption of CO were similar to those which have been described before. At sea level when the CO was administered in air no symptoms were noted until the (COHb) exceeded 15 per cent. From 15 to 25 per cent the symptoms consisted of mild to moderate post-

orbital headache, "giddiness" and slight dyspnea on standing erect or slight exertion and, on a few occasions, mild nausea. By contrast the symptoms at altitude were much more marked. For example, one of the subjects had experienced exposure to a pressure-altitude of 13,000 feet for periods of upwards of one hour on several occasions; no symptoms had been noted. Furthermore, his total blood concentration of COHb had been elevated to 20 per cent on several occasions with the development of only the mildest symptoms. However, the combination of exposure to 13,000 feet for one hour during which time the total (COHb) reached 21 per cent produced severe headache, loss of interest and marked tremor of the hands.

DISCUSSION. The linear uptake of CO by man will be maintained only during the early phases of exposure when the pressure gradient of CO between alveolar air and mixed venous blood has not been diminished significantly by the increasing back pressure of CO in the blood returning to the lungs. This phenomenon has been discussed fully by Forbes, Sargent and Roughton (1). The experiments reported here were so designed that the (COHb) finally attained did not exceed a quarter of its anticipated equilibrium value; thus the unpredictable effect of approaching equilibrium on the rate of CO uptake does not enter into the comparison of uptake rates under various conditions. The expression which relates the increase in (COHb) to the "CO exposure index" may be compared to similar expressions derived by Forbes *et al.*, and by Pace *et al.* (1, 3). All of these expressions are equivalent within the limits of accuracy of the analytical methods used, and together establish within rather precise limits the rate of uptake of CO by man during the earlier phases of exposure. The factor of exercise and the accompanying rise in ventilation has been defined by Forbes *et al.*, and in the studies of Pace *et al.* (1, 3).

Among the subjects studied by Forbes, Sargent and Roughton "one man rather consistently absorbed 10 to 15 per cent faster than the average and another about the same amount slower". The group which we studied did not contain any individual who deviated significantly from the average with respect to rate of CO uptake while breathing air at sea level or moderate altitude. If the explanation for the variation between individuals rests with differences in a , the ratio of tidal air to dead space of the lung, and b , the diffusion constant of the lung as suggested by Forbes, Sargent and Roughton, then the subjects studied in these experiments were fortuitously homogeneous with respect to these factors.

In a system containing O_2 , CO and hemoglobin the amount of COHb formed is inversely proportional to the partial pressure of O_2 ; this relationship has been found to hold true for equilibria reached both *in vitro* (5) and *in vivo* (2). Roughton showed that likewise the rate of COHb formation *in vitro* is similarly related to pO_2 (4). The experiments reported by Forbes *et al.*, and those recorded here show that roughly the same effect upon uptake rate occurs *in vivo*. An increase in pO_2 of an inspired mixture exerting a constant pCO depressed the rate of COHb formation. However, the effect of an increased pO_2 became evident only when sufficient time elapsed to make the difference detectable by presently available analytical technics.

Conversely, the rate of COHb formation during anoxia should be enhanced. However, the degree of anoxia (inspired pO_2 of 97 to 123 mm. Hg) which lends itself to study in man was apparently not sufficient in these short term experiments to make its effect detectable with respect to rate of CO uptake, although the symptoms produced by the combination of CO and altitude were increased noticeably in severity.

The reduction in total barometric pressure to the equivalent of 25,000 feet had no demonstrable effect on the rate of CO uptake. Forbes *et al.* found no effect at 40,000 feet in two experiments. This substantiates the general conclusion that within the limits of pressure-altitudes to which man may be exposed for any appreciable length of time the total pressure itself may be ignored in the prediction of the amount of CO absorbed.

SUMMARY

1. The rate of uptake of CO has been studied in 9 subjects at rest under a variety of partial pressures of oxygen in the inspired air.
2. The rate of uptake of CO may be predicted with considerable accuracy for sea level and altitude conditions by means of the following relation of the "CO exposure index" to the increase in (COHb): $\Delta (\text{COHb}) = (p\text{CO} \times \text{time} \times \text{minute respiratory volume}) \times 0.05$.
3. In accordance with the laws of combination of CO with Hb in the presence of O_2 , the rate of uptake of CO is inversely proportional to the partial pressure of O_2 .
4. Total barometric pressure plays no discernible rôle in the uptake of CO.
5. The effects of minimal anoxia due to altitude sum with the effects of small amounts of circulating COHb to produce moderately severe symptoms of anoxia.

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THE RELATIONSHIPS BETWEEN CARBON MONOXIDE, OXYGEN AND HEMOGLOBIN IN THE BLOOD OF MAN AT ALTITUDE¹

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The limits for permissible contamination of inspired air by carbon monoxide have been set, according to current military and industrial specifications, to preclude any undesirable concentrations of carboxyhemoglobin when equilibrium has been reached. The initial rates of uptake of CO by man have been studied recently by several groups (3, 7, 10). The cognate problem of the distribution of COHb, O₂Hb and reduced Hb and their related gas tensions has been studied in vitro with increasing precision from the original experiments of Douglas and the Haldanes to the most recent experiments of Roughton and Darling (2, 13). There is no information available, however, on the equilibria relations which obtain in vivo, especially at various pressure-altitudes. The study reported here was designed to furnish information on this phase of the general problem of CO intoxication.

METHODS. Three male subjects were studied: CF, a light smoker; JL, a heavy smoker; and RR, a non-smoker. The subject's blood level was elevated abruptly toward an estimated equilibrium value at the beginning of each experiment by administering a mixture of 0.7 to 2.0 per cent CO in air for 2 to 3 minutes (the "booster"). Then for periods ranging from 4 to 7 hours the subject lay at rest and breathed a mixture of CO, O₂ and N₂ through a close-fitting face mask from a demand regulator which metered the mixture delivered from a pressure tank.⁵ With one exception the experiments were carried on at the desired pressure-altitude in a decompression chamber. At regular intervals blood was drawn from the antecubital vein and analysed for CO. When consecutive analyses indicated that equilibrium had been reached, an indwelling needle was introduced into the brachial artery and two samples of arterial blood were withdrawn at approximately 30 minute intervals. This experimental design insured that at the time of obtaining the arterial samples no further increment in (COHb) was to be expected.

¹ The opinions and assertions contained herein are the private ones of the writers and do not reflect the official policies of the Navy Department or the naval service at large.

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⁵ These gas mixtures were furnished through the kindness of the National Bureau of Standards. Owing to the fact that the mixtures of CO in air were compressed with re-cycle air in a liquid air compressor, the oxygen content was less than that of air. The oxygen content of each tank was determined by the Haldane technic and the pressure-altitude in the decompression chamber adjusted to give the desired inspired pO₂. The percentages of CO were stated by the National Bureau of Standards to be 0.005, 0.010 and 0.015. Analyses by a colorimetric method for CO agreed with the stated concentrations (4).

The samples of venous blood were prevented from coagulating by the addition of dry potassium oxalate to produce a final concentration of 0.2 per cent. The samples of arterial blood were drawn directly into iced syringes containing 4 drops of liquid heparin with sodium fluoride in physiological saline (heparin 2 mgm./cc. and NaF to effect a final concentration of 0.05 per cent in sample). The following determinations were then carried out:

- a. pO_2 by the direct bubble method of Riley, Proemmel, and Franke (12);
- b. CO contents by the Scholander-Roughton microgasometric method with double quantities of blood (80 instead of 40 c.mm.) (16). With this modification duplicate analyses checked within 0.05 vol. per cent in the 36 consecutive samples of blood analysed in this study. The accuracy of the method is of this same high order;
- c. O_2 contents by the Roughton-Scholander microgasometric method (14);
- d. Total gas (CO) capacity by the photoelectric colorimeter of Andrews and Horecker (1). The accuracy of this instrument for the estimation of the CO capacity of the blood has been established in this laboratory by comparative gasometric analyses, and the correspondence of the two methods is 0.2 vol. per cent or better;
- e. CO_2 contents of the whole arterial blood by the manometric method of Van Slyke and Neill;
- f. pH s of the arterial blood by a glass electrode of the MacInnes and Belcher type (Cambridge Instrument Co.). The shielded assembly of the glass electrode, the internal silver-silver chloride electrode and the calomel reference electrode, and all the solutions were maintained at a constant temperature of $37^\circ C$. in a warm air bath to eliminate all temperature gradient potentials. The glass electrode circuit was standardized with 0.05 M potassium acid phthalate and calibrated with Sørensen phosphate buffers. The pH of these solutions at $37^\circ C$. was kindly calculated for us by Dr. W. J. Hamer of the National Bureau of Standards.

RESULTS. The hourly blood CO levels are presented in figure 1. It will be noted that the blood concentrations of CO had reached constant levels at least one hour before the samples of arterial blood were drawn.

The pertinent data for each experiment are presented in table 1.

DISCUSSION. Haldane and his collaborators showed originally that the hemoglobin in a solution saturated with a mixture of CO and O_2 was distributed between $COHb$ and O_2Hb according to the following expression (Haldane's first "law"):

$$\frac{(CO)}{(O_2)} = \frac{(COHb)}{(O_2Hb)} = \frac{M_{pCO}}{p_{O_2}} \quad (A)$$

where M = the relative affinity constant of Hb for CO compared to O_2 , and (CO) and (O_2) = gas contents of the blood in volumes per cent, and $(COHb)$ and (O_2Hb) = per cent saturation of the total hemoglobin (2).

Roughton and Darling have shown recently that the expression (A) holds true both for hemoglobin solutions and for whole blood even when an appreciable amount of reduced hemoglobin (Red. Hb) is present in the system (13). This

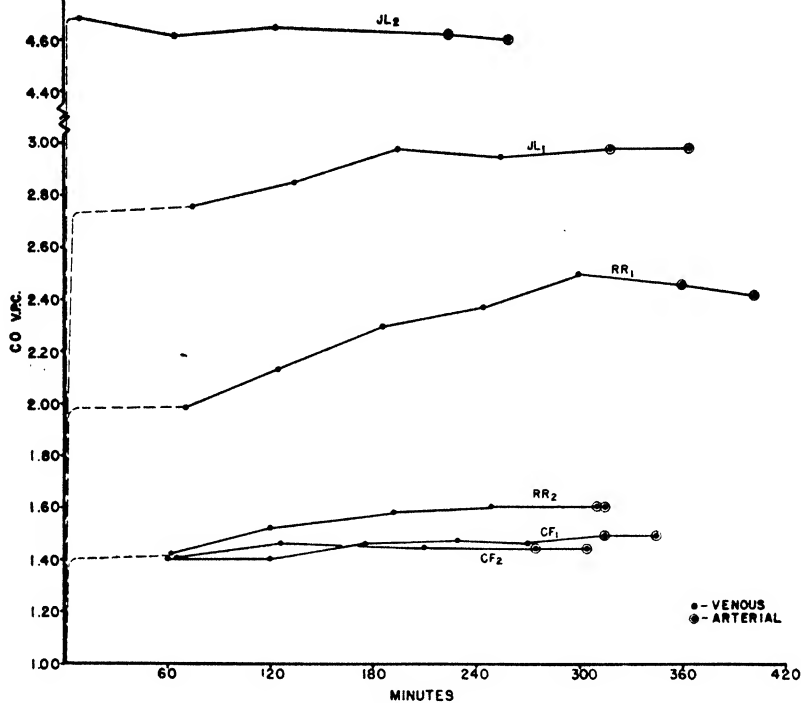


Fig. 1. Blood CO during prolonged exposure.

TABLE 1
Blood and gas studies during CO equilibrium

SUBJECT AND EXPT. NO.	MINUTES OF EXPOSURE	EQUIVALENT ALTITUDE (THOUS- ANDS OF FEET)	INSPIRED pO_2 MM Hg	INSPIRED PER CENT CO	ARTERIAL pO_2 MM Hg	ARTERIAL pCO MM Hg	M or $pO_2 \times (CO)$ $pCO \times (O_2)$	$MpCO$ or $pO_2 \times \frac{[CO]}{[O_2]}$ $pO_2 + MpCO$	ARTERIAL O_2			ARTERIAL CO			ARTERIAL $O_2 + CO$			$100 \times (O_2Hb) + (COHb) + (Red.Hb)$	ARTERIAL pCO_2 MM Hg	ARTERIAL CO_2 CONTENT V.P.C.	pH_s	
									Content V.P.C.	Capacity V.P.C.	Saturation P.C.	Content V.P.C.	Capacity V.P.C.	Saturation P.C.	Content V.P.C.	Capacity V.P.C.	Saturation P.C.					
CF ₁	315	10.0	108	0.005	56	0.024	214	5.1	61.1	16.4	18.5	88.6	1.49	20.0	7.5	17.9	20.0	89.5	82.5	33	46.8	7.485
	345	10.0	108	0.005	59	0.024	224	5.3	64.3	16.5	18.5	89.2	1.49	20.0	7.5	18.0	20.0	90.0	80.6	36	46.9	7.470
CF ₂	275	10.0	109	0.005	54	0.025	192	4.8	58.8	16.2	18.6	87.2	1.44	20.1	7.2	17.7	20.1	87.8	80.6	36	46.4	7.440
	305	10.0	109	0.005	58	0.025	204	5.1	63.1	16.3	18.3	89.0	1.44	19.7	7.3	17.8	19.7	90.0	82.6	33	46.6	7.450
JL ₁	320	15.4	88	0.010	40	0.048	182	8.7	48.7	13.8	17.4	79.3	2.97	20.4	14.7	16.8	20.4	82.3	67.7	37	44.7	7.470
	365	15.4	88	0.010	40	0.048	184	8.7	48.7	13.7	16.7	82.0	2.97	19.7	15.2	16.7	19.7	84.7	69.5	29	44.0	7.510
JL ₂	227	6.5	126	0.015	69	0.106	202	21.8	90.8	14.5	14.7	98.5	4.61	19.3	23.8	19.1	19.3	98.9	75.1	36	48.3	7.415
	260	6.5	126	0.015	66	0.106	208	21.4	87.4	14.2	14.9	95.7	4.59	19.5	23.6	18.8	19.5	96.7	78.0	38	48.1	7.405
RR ₁	360	10.0	107	0.010	64	0.066	197	11.0	75.0	14.3	14.9	96.2	2.45	17.3	14.2	16.8	17.3	96.8	82.6	35	47.0	7.430
	400	10.0	107	0.010	63	0.056	191	10.6	73.6	14.3	14.9	96.2	2.41	17.3	13.9	16.7	17.3	96.5	82.6	32	47.2	7.440
RR ₂	310	10.0	106	0.005	54	0.025	225	5.7	59.7	15.1	17.0	88.8	1.60	18.6	8.6	16.7	18.6	89.7	81.3	37	46.7	7.415
	313	10.0	109	0.005	54	0.025	223	5.7	59.7	15.2	17.0	89.4	1.60	18.6	8.6	16.8	18.6	90.3	81.7	40	47.1	7.415

demonstration in vitro also furnished experimental confirmation of Haldane's second "law": in blood exposed to O_2 at a partial pressure pO_2 and to CO at a partial pressure pCO the total hemoglobin saturation,

$100 \times \frac{(COHb + (O_2Hb))}{(COHb) + (O_2Hb) + (Red. Hb)}$, is the same as it would be in the absence of CO, if pO_2 then equaled $pO_2 + MpCO$. These relations imply likewise that

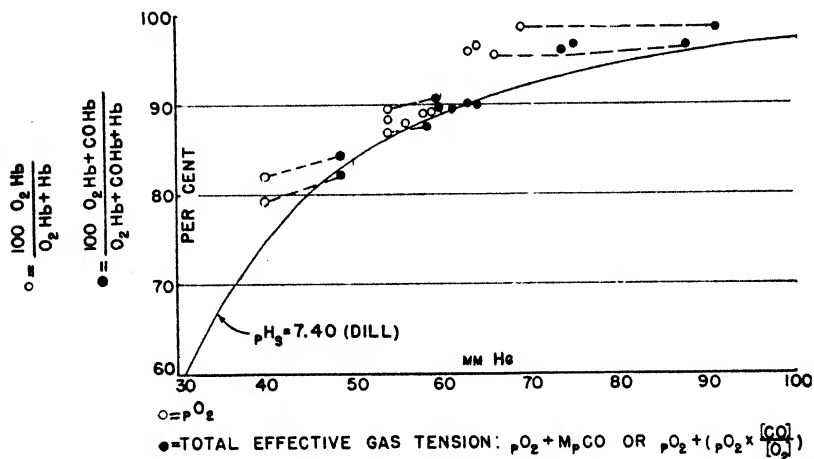


Fig. 2. The Haldane effect

the following pairs of functions may be described by the same standard oxy-hemoglobin dissociation curve:

- (I) pO_2 and $100 \times \frac{(O_2Hb)}{(O_2Hb) + (Red. Hb)}$ in the absence of CO,
- (II) $MpCO$ and $100 \times \frac{(COHb)}{(COHb) + (Red. Hb)}$ in the absence of O_2 ,
- (III) $pO_2 + MpCO$ and $100 \times \frac{(O_2Hb) + (COHb)}{(O_2Hb) + (COHb) + (Red. Hb)}$

The experimental data of this present study provide the means for putting Haldane's second "law" to the test in man. An inspection of figure 2 shows that when the total effective gas tension is plotted against the total hemoglobin saturation (pair III above) the experimental points (solid circles) do in fact fall along the standard oxyhemoglobin dissociation curve. These findings confirm Haldane's second "law" when applied to equilibrium conditions existing in vivo.

Haldane's third "law" first drew attention to what is now termed the "Haldane effect": the presence of COHb shifts the oxygen dissociation curve of the remaining hemoglobin to the left (5). This "Haldane effect" has been re-examined and confirmed recently by Roughton and Darling in a series of precise experiments in

vitro from which they have derived "some profitable simplifications in the development of the theory" (13). Their treatment of the data and the theory showed that the displacement of the oxyhemoglobin dissociation curve could be predicted through the use of the fundamental equation (A) and a standard oxyhemoglobin dissociation curve without knowledge of M or pCO or recourse to the Hill-Barcroft equation. Their assumption that "the effect of COHb on the O₂-dissociation curve in vivo should be quantitatively the same as the experimentally observed effect in vitro" has been confirmed in this present study: the open circles in figure 2 (the in vivo dissociation of oxyhemoglobin in the presence of varying amounts of COHb) indicate by their displacement the "Haldane effect", and coincide with the calculated curves of Roughton and Darling (v. fig. 1 of their paper).

Although Roughton and Darling showed that a definition of the individual values of M and pCO were not needed to calculate the effect of COHb on the oxyhemoglobin dissociation curve, nevertheless, a knowledge of these factors adds to an understanding of equilibrium conditions in vivo. The data which are recorded in table 1 afford an opportunity to calculate these factors.

pCO—The arterial pCO could not be determined directly. However, under the conditions of equilibrium existing in these experiments, the arterial pCO may be assumed to have been equal to the alveolar pCO, which could be calculated readily from the pCO of the inspired mixture (alveolar pCC = inspired per cent CO \times (P_{bar} - 47)).

M—When the derived value for arterial pCO is substituted in a rearrangement of the fundamental equation (A) together with the other terms which have been determined directly, then

$$M = \frac{pO_2 \times (COHb)}{pCO \times (O_2Hb)} \quad (A_1)$$

The validity of the assumption that arterial pCO = alveolar pCO gains support from the fact that the average value of M calculated on this basis (table 1) is 204 ± 10 per cent as compared with 210 ± 2.5 per cent determined in vitro by Sendroy, Liu and Van Slyke (15). This correspondence provides evidence that the same equilibrium is attained by the human subject as that which obtains in the tonometer.

The fundamental Haldane equation,

$$\frac{MpCO}{pO_2} = \frac{(COHb)}{(O_2Hb)}, \quad (A)$$

can be rearranged after substituting

$$(O_2Hb) = (Tot. Satn) - (COHb) \quad (B)$$

as follows:

$$(COHb) = \frac{MpCO}{pO_2 + MpCO} \times (Tot. Satn) \quad (C).$$

Equation (C) is a useful expression which lends itself to the rapid estimation of the amount of COHb to be found in the blood of man in CO-equilibrium at any altitude through the use of generally accepted average values for M, pO_2 and (Tot. Satn). The use of equation (C) is demonstrated best by working through a sample calculation:

Given the problem of calculating the amount of COHb in the blood of a subject exposed to 0.008 per cent CO in air at a pressure-altitude of 10,000 feet ($P_{bar} = 523$ mm. Hg) until equilibrium is reached, the following values would be substituted in equation (C)

$$pCO = (523-47) \times 0.00008 = 0.038 \text{ mm. Hg}$$

$$M = 210 \text{ (Sendroy, Liu and Van Slyke, 14)}$$

$$pO_2 = 61 \text{ mm. Hg (from Boothby's curve, 6)}$$

(Tot. Satn) = 92 per cent (read off a standard oxyhemoglobin dissociation curve (11) at a tension of $pO_2 + MpCO = 69$ mm. Hg).

whereby (COHb) = 10.7 per cent

This calculation involves assumptions and values which have been tested only at rest and during mild exercise.

Evidence regarding the accuracy of this simplified method of estimating the equilibrium value of COHb at altitude is furnished by comparing values so calculated with those determined in our experiments (table 1):

Per cent saturation COHb			
Expt. no.	Determined	Calculated	Δ per cent COHb
CF ₁	7.5	7.0	+ 0.5
CF ₂	7.2	7.2	0.0
JL ₁	15.2	16.4	- 1.2
JL ₂	23.6	23.2	+ 0.4
RR ₁	14.2	15.1	- 0.9
RR ₂	8.6	7.0	+ 1.6

The average deviation of the calculated from the determined value is less than 0.8 per cent.

Since the basic Haldane equation (A) describes the experimental data at all pressure-altitudes studied, it appears that variations in total barometric pressure do not affect equilibrium relations. This finding is consonant with the related observations that the uptake of CO by man at altitude is a function of pCO and pO_2 but not of P_{bar} (3, 7, 10).

There is no evidence in the data presented here to indicate that the presence of appreciable amounts of COHb in the blood of smokers changes the affinity of hemoglobin for CO or O_2 . For example, the blood of subject RR was found to contain 0.2 vol. per cent or less of CO whenever examined in basal state; on the other hand, the blood of subject JL had a constant CO content of 1.2 to 1.5 vol. per cent. Nevertheless, the affinities of these two individual hemoglobins for CO and O_2 were found to be virtually identical.

An estimate of the effect of a given concentration of COHb on certain physiological functions in man at altitude has been made difficult by the discordant results and conclusions reported by the several investigators who have examined this problem (8, 9, 17). The data recorded in this study complicate the practical problem further by indicating that the duration of exposure to a given concentration of COHb is a factor which cannot be ignored. For example, in two of the experiments in this series there were noted significant symptoms which appeared only after considerable time had elapsed. In experiment RR₁, the subject noted steadily increasing headache and recurrent nausea during the final 3 to 4 hours of exposure; in experiment JL₁, no symptoms were noted during the first hour, but thereafter there appeared headache which became progressively more severe, increasing and almost constant nausea, mental confusion, restlessness, pallor, cold extremities and a state of mild shock. These symptoms increased in severity as time passed although there was little if any change in (COHb), (O₂Hb) or the blood gas tensions during the course of the experiment. Thus, the symptoms produced by CO were related not only to the concentration of COHb but also to the duration of exposure.

SUMMARY

1. Three male subjects have been studied at varying pressure-altitudes while in equilibrium with inspired gas mixtures containing from 0.005 to 0.015 per cent carbon monoxide.

2. The distribution of COHb, O₂Hb and reduced Hb and their related gas tensions confirm in vivo the fundamental "laws" first defined by Haldane for the equilibria obtaining in vitro.

3. A simple rearrangement of the Haldane equation makes possible an accurate prediction of the amount of COHb obtaining when man is in equilibrium with a CO-contaminated atmosphere at any given altitude.

The equation,

$$(\text{COHb}) = \frac{\text{MpCO}}{p\text{O}_2 + \text{MpCO}} \times (\text{Total hemoglobin saturation}),$$

requires only that the percentage of CO in inspired air and the pressure-altitude be known. The remainder of the terms may be read from standard values, tables and curves; e.g., $M = 210$, $p\text{O}_2$ = average alveolar tension at the given pressure-altitude, and (Total hemoglobin saturation), related to the tension of $p\text{O}_2 + \text{MpCO}$, may be read off the standard oxyhemoglobin dissociation curve.

4. The value of the relative affinity constant of Hb for CO compared to O₂ was found to be 204 ± 10 per cent in these experiments.

5. The total barometric pressure has been found to play no rôle in the distribution of CO and O₂ at equilibrium.

6. The hemoglobin of individuals who smoke appears not to differ from that of non-smokers in its affinity for CO and O₂.

7. The symptoms produced by CO are proportional not only to the blood concentration of COHb but also to the duration of exposure to a given concentration.

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THE EFFECT OF LOW CONCENTRATIONS OF CARBOXYHEMOGLOBIN ON THE "ALTITUDE TOLERANCE" OF MAN¹

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When the human subject has absorbed enough CO to have converted one-quarter to one-third of his available circulating hemoglobin to COHb, the effects of this degree of CO anoxia on a variety of physiological functions become evident. By contrast the man who smokes moderately to heavily carries on without appreciable handicap with from one-twentieth to one-fifteenth of his hemoglobin bound to CO. It seems reasonable to suspect that between these extremes there lies a degree of carbon monoxide anoxia which produces undesirable effects on delicate mechanisms without subjective awareness of any handicap. This possibility is of paramount importance in aviation where man may be exposed to the additive effects of both CO and anoxic anoxia in addition to a variety of other physiological stresses. Since absolute freedom from contamination of ambient air with CO is frequently difficult to achieve, it becomes important to measure the effects of small increments in COHb upon function in man already under the stress of mild anoxic anoxia. The difficulty inherent in all such measurements arises from the fact that "altitude tolerance" cannot be defined more precisely than by describing the alterations which occur in one or more of a whole series of physiological functions. Thus, for example, scotopic vision may show measurable impairment at low altitudes while auditory function may be well maintained during severe anoxia. Between these two levels various visual, psychomotor, circulatory and respiratory functions deteriorate serially as the barometric pressure falls. One of the functions which is impaired by exposure to relatively mild degrees of anoxia is the flicker fusion frequency (FFF); this is the critical frequency in cycles per second (c.p.s.) at which a flickering light appears to be steady.

This paper reports a study of the effect of low concentrations of COHb in man at altitude by measuring the changes in FFF as an index of anoxia.

METHODS. The subjects of this study were 5 males, each 18 years old, who had been examined and accepted for flight training.

The measurement of FFF was made by means of an oscilloscope manufactured by the Electronic Supply Company, Worcester, Mass. The instrument consisted of a 5 mm. target which was viewed by the subject through a 12-inch-long tube lined to eliminate reflection. The right eye was used and the left covered. The target was a neon glow tube operated by suitable circuits to insure a constant

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light intensity. The frequency of the pulsating current which drove the glow tube was adjustable to within 0.2 c.p.s. The rates of flicker were calibrated against an audio-oscillator by means of a 1 inch cathode ray oscilloscope incorporated in the circuit. All experiments were carried out within a decompression chamber in which the background illumination was kept low and constant. The technic of measurement was executed as follows: the subject fixed the target while the rate of flicker was well above his capacity to distinguish separate oscillations. The rate was then decreased steadily until he observed the onset of flicker. The rate was then abruptly increased and the measurement repeated. Three such determinations were carried out in quick succession within 10 to 15 seconds. The subject then withdrew his eye from the viewing tube. The series of three determinations was repeated in 2 to 3 minutes. The FFF was expressed as the average of the 3 rapidly repeated measurements. The reproducibility of the method depends upon careful training of the subject until he recognises a constant end-point in the discrimination of flicker. In subjects so trained the three measurements performed in rapid succession are usually equal and only exceptionally vary among themselves by more than 0.2 c.p.s. while the average FFF does not change more than 0.2 to 0.5 c.p.s. over a period of several hours.

Many observations in this laboratory have established the fact that significant lowering of the FFF occurs at a pressure-altitude which is characteristic for the individual studied (5). We have selected arbitrarily a decrement in FFF of 1.5 c.p.s. or greater as significant of deterioration after standard exposure of 20 minutes to the simulated pressure-altitude. The standard test is completed by the administration of aviator's breathing oxygen (99.5 per cent) for 10 minutes at the same test altitude. Characteristically, after this period of oxygen inhalation the FFF returns to the sea level frequency established before the induction of anoxia. The standardisation of the 5 subjects studied is presented in table 1. It will be noted that in 2 of the subjects, FFF deteriorated at pressure-altitudes beginning at 10,000 feet; 2 showed depressed FFF beginning at 11,000 feet, and one subject showed significant change beginning only at 12,000 feet. Furthermore, after the administration of oxygen the FFF returned to the sea level threshold.

In studying the effects of CO on "altitude tolerance", the subjects were administered suitable proportions of CO in air (0.15 to 0.2 per cent). The gases were mixed in a Tissot spirometer, transferred to an impervious rubber bag, and delivered to the subject through respiratory valves and a mouth-piece. The mixtures were administered for a period sufficient to produce the required increments in COHb (3). CO-air mixtures were administered first at sea level, and in subsequent experiments the CO exposures were carried out in a decompression chamber at simulated pressure-altitudes of 5,000 and 6,000 feet. One group of measurements was made after 15 to 25 minutes' exposure to CO and altitude when the FFF was determined and a sample of blood drawn for analysis. In another group the flickering target was set at a frequency 1.0 c.p.s. less than the sea level FFF. After 10 minutes' exposure to altitude and CO the subject viewed the target briefly every two minutes until he reported that the flicker had vanished (12 to 20 min.); a complete FFF measurement was made then and

a sample of blood obtained. This second group of experiments furnished an estimate of the threshold concentration of COHb at which FFF was first impaired at the pressure-altitude under study. At the completion of each experi-

TABLE 1
"Altitude tolerance" breathing ambient air

SUBJECT	PRESSURE ALTITUDE (IN THOUSANDS OF FEET)	FFF LEVELS IN C.P.S.		
		Sea level	After 20 minutes' exposure at altitude	After 10 minutes on O ₂ at altitude
D	7	48.8	48.5	—
	8	49.5	49.3	—
	9	49.5	49.3	—
	*10	49.3	45.5	49.4
	*11	49.4	47.2	49.3
	*12	47.9	45.0	46.8
L	7	49.3	49.5	—
	8	48.0	47.8	—
	9	47.5	47.8	—
	10	47.6	47.5	—
	*11	47.7	45.4	47.2
	*12	49.3	47.4	49.5
M	7	47.0	47.0	—
	8	45.5	45.5	—
	9	45.5	45.5	—
	*10	47.5	45.4	47.4
	*11	47.8	45.3	47.8
	*12	49.4	47.4	48.6
N	7	49.3	49.0	—
	8	49.5	49.5	—
	9	49.3	49.0	—
	10	49.6	49.4	—
	11	49.5	49.1	49.2
	*12	51.8	50.2	52.0
S	7	48.0	47.8	—
	8	46.5	46.5	—
	9	46.5	46.5	—
	10	47.6	47.2	—
	*11	47.8	45.1	47.4
	*12	49.4	47.5	49.1

* Signifies exposures exceeded "altitude tolerance."

ment, oxygen was administered to the subject for 10 minutes and the recovery FFF measured. No subject was exposed to anoxia twice on the same day.

The concentration of COHb in samples of cutaneous blood obtained from the finger tip was estimated by the microgasometric method of Scholander and Roughton (7). The hemoglobin gas capacity was assumed to be 19.5 vols per

cent. The effective concentration of COHb was expressed as the increment above the concentration existing before exposure (1.0 to 3.5 per cent).

RESULTS. The results of these experiments are presented in tables 1 and 2. It may be noted from table 1 that none of these subjects showed any deterioration of FFF after 20 minutes' exposure to a pressure-altitude of 9,000 feet. Furthermore, at sea level, increases in blood concentrations of COHb from 13.8 to 17.4 per cent produced no change in FFF during the acute exposures studied. However, the data recorded at 5,000 and 6,000 feet furnish evidence that relatively

TABLE 2

Relation of increment in COHb per cent to effect on FFF

Each value is the per cent COHb determined in the subject at the end of an individual experiment.

SUBJECT	SEA LEVEL		5,000 FEET		6,000 FEET	
	FFF Depressed	FFF Constant	FFF Depressed	FFF Constant	FFF Depressed	FFF Constant
D		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
		8.4	8.7*	10.5	9.2	
		9.0	10.7		9.3	
		17.4	13.8		12.3*	
L		10.0	5.1*	4.5	8.8	8.7
		11.4	6.1*	6.7	16.9	
		13.8				
M		10.5	7.2		10.3	
		11.1	9.3*		10.8*	
		14.9				
N		7.3	7.1*	4.0	9.2	5.6
		7.4			10.2*	
		15.5				
S		8.2	9.2*	7.3	6.9*	7.7
		10.1			10.7	
		15.5			14.8*	

* Measured at first evidence of depression in FFF.

small increments in the amount of circulating COHb (Δ per cent COHb) produced an appreciable depression in "altitude tolerance" when measured by the deterioration in the FFF.

Reference to table 2 indicates that a combined exposure to a 5,000 or 6,000 feet pressure-altitude and a Δ COHb of 5 to 9 per cent produced an impairment of FFF although either of these stresses alone did not affect FFF. The data, which do not permit more than an approximate estimate of the loss of "altitude tolerance", do indicate, however, that a Δ COHb of 8 to 10 per cent above resting values effected a lowering of "ceiling" of 4,000 feet or more. For example, the subject N when exposed for 20 minutes to a simulated pressure-altitude of 11,000 feet maintained FFF without change. At 5,000 feet this subject exhibited

an abrupt deterioration in FFF when his blood COHb had increased 7.1 per cent; the increment in blood COHb had depressed FFF in an amount to the effect provided by simple anoxia at 12,000 feet, or, conversely, an increment of 7.1 per cent COHb had converted an actual altitude of 5,000 feet to an equivalent altitude of 12,000 feet with respect to the function under test.

DISCUSSION. An estimation of the effect of a given physiological stress upon man is made by necessity in most instances by measuring the changes produced in one or more relatively isolated functions. Aside from the difficulties introduced by the uncertainties inherent in any functional measurement there is encountered frequently the additional problem arising from the widely differing degrees of influence of a single stress upon various functions. This fundamental difficulty is met inescapably in the present problem of the effect of CO upon man. For example, Haldane in his pioneer experiments found that "when 30 per cent of his hemoglobin was combined with carbon monoxide the symptoms were comparatively mild at rest" (2). Asmussen and Chiodi in a study of the effect of various forms of anoxia upon the respiration and circulation exercised their subjects heavily (O_2 consumption of 1.5 to 2 l./min.) while 20 to 30 per cent of their hemoglobin had been combined with CO; they make no direct reference to the development of symptoms or handicap but the degree of work was continued for a number of minutes so that it may be inferred fairly that the subjects were able in any case to carry on heavy work despite a high degree of CO anoxia (1). From these observations it might be concluded that when a quarter to a third of his circulating hemoglobin had been prevented by CO from participating in normal O_2 transport, man could bring to bear compensatory mechanisms which maintained the efficiency of his over-all functioning. That this would be a fallacious conclusion was demonstrated forcibly by McFarland, Roughton, Halperin and Niven (6). These investigators employed a delicate function, visual discrimination, to measure and compare the effect of anoxia produced by reduced O_2 pressure and by inhalation of CO. They report that "deep inhalation of the smoke from a single cigarette causes an increase in the carboxyhemoglobin saturation of almost 2 per cent. Although previously such a small amount was considered totally insignificant, it nevertheless caused a distinct impairment of visual sensitivity. After three cigarettes, the blood COHb was 4 per cent and the effect on one subject's visual sensitivity was equal to that at an altitude of almost 8,000 feet". Again, these investigators compute, for example, that at an effective pressure-altitude of 6,000 feet the presence of 10 per cent COHb depresses visual discrimination equivalent to the depression produced by an exposure to an altitude of 12,000 feet breathing air. It becomes apparent from these several studies that the effect of CO, especially during exposure to mild altitude anoxia, may be either insignificant or impressive depending upon what type of performance is studied.

The studies reported here, which employed another relatively delicate visual function (FFF) to measure the effect of CO at altitude, agree in general with the findings of McFarland *et al.* (6). The absorption of moderate amounts of CO (5 to 10 per cent COHb) produced deterioration in visual processes which may be interpreted reasonably to reflect the handicap under which the cerebral processes

labor. The measurement of visual discrimination used by McFarland *et al.*, however, suffers as a practical tool to a certain extent from its extreme delicacy and susceptibility to such minor stresses as smoking. One conclusion which might be deduced from their findings is that aviators flying at relatively low altitudes must either abstain from smoking or be protected by the addition of O₂ to their inspiratory mixture. In this regard, the use of FFF as a measure of the CO effect has certain practical advantages to recommend it. The extensive experience of latter years has shown that man can fly at altitudes up to 8,000 to 10,000 feet with comparative safety while breathing air. Above this altitude experiential and theoretical evidence has indicated the need for the administration of an oxygen-enriched breathing mixture. By chance, detectable deterioration in FFF occurs first at pressure-altitudes which correspond to the altitudes at which experience has dictated the necessity for added oxygen (9,000 to 12,000 feet). The FFF has provided, therefore, a useful measure of the effect of small increases in the amount of circulating COHb in terms of the lowering of "ceiling", or conversely raising of the "physiological altitude", in terms of the effect of exposure to the maximal altitudes at which added oxygen is not usually supplied to the aviator.

If the lessons to be learned from the studies of the CO-sensitive functions are to be applied directly to the evolution of standards for permissible contamination of air by CO at altitude, then there must be assumed an uncompromising attitude toward relaxation of CO specifications. For example, the inhalation of ambient air containing 0.008 per cent CO at 10,000 feet will, when equilibrium is reached, convert 10 per cent of the available hemoglobin to COHb (4). With this concentration of COHb there will appear unequivocal impairment of both visual discrimination and FFF at altitude which, insofar as these means of measuring function are applicable, indicates that even so slight a contamination of air with CO as 0.008 per cent is, at altitude, an additional physiological stress to be avoided.

SUMMARY

1. By means of measuring a sensitive visual function, the critical flicker fusion frequency, which is impaired by mild anoxia (9,000 to 12,000 ft. pressure-altitude) the effect of small amounts of COHb on man has been studied at altitude.

2. Increments in COHb of the order of 5 to 10 per cent resulted in appreciable deterioration of FFF at altitudes which alone did not affect the FFF (5,000 and 6,000 ft.).

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BLOOD SUGAR LEVELS AND CARBOHYDRATE ADMINISTRATION IN HUMAN SUBJECTS DURING PROLONGED EXPOSURES TO MODERATELY LOW ALTITUDES

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The recent trend in military aviation toward airplane flights of longer duration has focussed attention on the fact that relatively little is known of the physiological responses of the unacclimatized individual during prolonged exposures to moderately low altitudes. Long range missions in pressurized or non-pressurized aircraft have made it necessary to consider two factors which may affect well being and efficiency in flight; namely, the cumulative anoxia which may result from too prolonged exposure to even low altitudes, and the detrimental effects of inadequate nutrition before and during flight. Evidence accumulates to indicate that nutritional factors may significantly influence the degree of response of an individual to oxygen lack. Ivy *et al.* (1), King *et al.* (2), and Barach *et al.* (3) have recently discussed the bio-chemical implications regarding the effect of the proximate principles on anoxia and have given quantitative evidence for the ameliorative effect of high carbohydrate administration, in contrast to high protein, on anoxic tolerance.

Experiments demonstrating increased altitude tolerance with high carbohydrate have generally involved relatively short exposures, and have been done at altitudes (15–23,000 ft.) where military personnel already use supplementary oxygen as required by directive. Any gain in altitude tolerance with carbohydrate would be of greater practical significance, however, at those altitudes below the critical anoxic zone, where supplementary oxygen is not ordinarily taken, and where the continuous use of oxygen for many hours presents a very practical problem. The academic and practical implications of increased altitude tolerance under these conditions warrant a detailed study of carbohydrate metabolism as well as the determination of the effects of carbohydrate ingestion, in individuals exposed to moderately low altitudes for prolonged periods. The present experiments, representing one phase of a project investigating the physiological requirements of long range missions, will be concerned specifically with the determination of the blood sugar in men exposed to simulated altitudes of 8000 and 10,000 feet for 10 hour periods without supplementary oxygen. The effects of high carbohydrate administration to individuals maintained under these conditions will also be described.

PROCEDURE. Six subjects (military personnel), ranging from 20 to 35 years of age, were used in the experiment for a grand total of 102 man runs. Each subject received a total of at least eight runs at ground level and a similar number at altitude. Each subject underwent one pressure chamber flight weekly. The

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individuals reported to the laboratory on the day of the experiment in the post-absorptive state, their previous meal having been taken at the mess hall approximately 12 to 14 hours the evening before. No attempt was made to control the amount and composition of the evening meal. Fasting capillary blood was drawn in duplicate and prepared for blood sugar analysis using the Horvath-Knehr modification of the Folin-Malmros micromethod (4). In the latter part of the experiment Nelson's modification of the Somogyi method (5) for true blood sugar was used. After basal measurements were taken, the subjects were fed a standard breakfast of army K ration, (table 1) with 250 cc. of water, which was consumed within 20 minutes. Upon completion of breakfast, the pressure chamber was brought to the desired altitude (rate 3000 ft./min.) and the subject maintained there at rest for 8 to 10 hours breathing ambient air with no subsequent food but with water allowed "ad libitum". The temperature in

TABLE 1
*Composition and estimated nutritive value of standard K-ration breakfast**

COMPOSITION	AMOUNT	CALORIES
Protein.....	29.2 grams	117
Fat.....	31.7 grams	291
Carbohydrate.....	119.0 grams	476
Calcium.....	295 mgm.	
Iron.....	6.6 mgm.	
Vit. A (IU).....	3138	
Thiamin.....	0.55 mgm.	
Riboflavin.....	0.7 mgm.	
Niacin.....	6.9 mgm.	
		884 (Total Calories)

* The above calculations are based on the most recent data available to the Q. M. C. Subsistence Research and Development Laboratory and the Subsistence Division, O. Q. M. G.

the chamber was approximately the same from run to run ($24 \pm 2^\circ\text{C}.$). The chamber was well ventilated at all times. Capillary blood was taken at $1\frac{1}{2}$ hourly intervals throughout the entire experimental period. The procedure was identical for control runs except that the chamber was kept at ground level (800 ft.).

The tolerance of each subject to carbohydrate was tested by the ingestion of 70 or 150 grams of commercial dextrose in 500 cc. solution flavored with a crystal of citric acid. The sugar was given at the approximate mid-point of the experiment; approximately 5 hours after the standard breakfast and start of observations. Capillary blood samples were taken for 4 successive half-hourly periods and at hourly intervals thereafter. Only the Folin-Malmros method for blood sugar was used in the dextrose tolerance experiments. Control tests, both at altitude and ground level, were accomplished by the administration of a saccharine solution.

RESULTS. Analysis of the data (table 2, fig. 1) indicates that the mean values

TABLE 2

Comparison of mean capillary blood sugar values obtained in six subjects at altitude and ground level over corresponding time intervals

ALT. (FT.)	METHOD	FASTING BLOOD SUGAR	TIME (HRS.) AFTER K-RATION BREAKFAST						
			1	2.5	4	5.5	7	8.5	10
G. L. (800)	Folin- Malmros	102* ± 18 (78)†	116 ± 22 (35)	96 ± 17 (32)	95 ± 23 (37)	90 ± 16 (23)	88 ± 14 (23)	89 ± 15 (15)	90 ± 11 (15)
8,000- 10,000			117 ± 18 (39)	105 ± 17 (38)	99 ± 14 (36)	95 ± 15 (20)	101 ± 16 (20)	99 ± 17 (15)	101 ± 17 (15)
Standard error of difference of means.....			5.0	4.4	4.7	4.7	4.6	7.2	6.4
G. L. (800)	Nelson- Somogyi	88 ± 18 (19)	104 ± 19 (8)	80 ± 6 (8)	82 ± 8 (8)	84 ± 8 (8)	86 ± 3 (8)	78 ± 12 (8)	76 ± 11 (8)
8,000- 10,000			96 ± 14 (9)	76 ± 10 (8)	78 ± 15 (9)	77 ± 11 (8)	77 ± 13 (8)	73 ± 10 (8)	77 ± 11 (8)
Standard error of difference of means.....			8.5	5.0	5.8	4.0	5.1	6.0	5.7

* Mean blood sugar ± standard deviation.

† Total number of determinations.

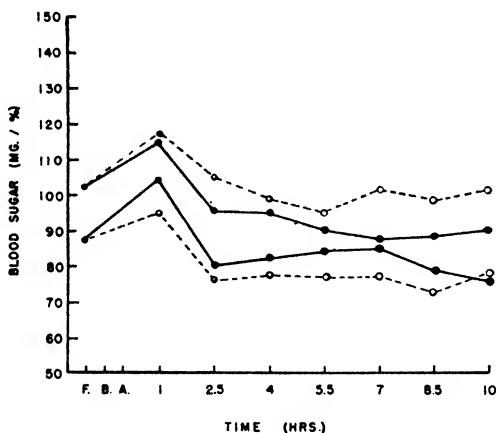


Fig. 1. Mean blood sugar level, for all subjects combined, at ground level and altitude (8000 and 10,000 ft.) F, B, A refer to initial fasting state, consumption of the standard breakfast, and ascent to altitude respectively. The experiment proper begins at A.

Solid lines refer to ground level; dotted lines to altitude. The two upper curves represent values obtained with the Folin-Malmros method; the two lower curves values, with the Nelson-Somogyi technique.

for the blood sugar as determined in six resting subjects at ground level and at altitude for 10 hour periods, following a K ration breakfast (table 1) with no subsequent food, were not significantly different at corresponding time intervals. The mean values obtained with the Somogyi method were consistently lower than those with the Folin method. The mean difference between both methods, for the entire 10 hour period, was approximately 15 mgm. per cent. In either case, the mean blood sugar level rose following the standard breakfast, declined to a level approximating that of the fasting state in several hours, and was thereafter maintained with little fluctuation within normal limits for the remainder of the experimental period. Although variation in blood sugar level did occur among various subjects and in the same subject from run to run, in no individual could the blood sugar level be interpreted as indicating a true hypoglycemic or hyperglycemic trend.

The ingestion of 70 or 150 grams of dextrose at the approximate mid-point of the 10 hour period was found to induce the development of "hypoglycemic" reactions in 5 out of 6 subjects approximately 50 per cent of the total number of times tested (table 3). These hypoglycemic episodes usually began within 3 to 5 hours after ingestion of the sugar and occurred with approximately equal frequency at either ground level or altitude. The seizures were characterized by subjective sensations of nervousness, impending danger, paresthesia, weakness, and hunger, and objective manifestations of pallor, sweating, tremulousness, coldness of the extremities, and a tendency, in some, toward a drop in oral temperature. The duration and severity of the reaction varied among individuals and in the same individual. Severity, however, was generally as marked at altitude as at ground level and as pronounced with the smaller as with the larger dose. The larger dose (150 grams) tended to delay the onset of the hypoglycemic reactions.

Comparison of the mean blood sugar values (table 4) and the resulting tolerance curves (fig. 2) after ingestion of glucose indicates that the return of the blood sugar level to the pre-ingestion value occurred at approximately the same time at altitude and ground level with the same dose of sugar. The time required for the blood sugar level to reach the preingestion value was significantly greater with the larger than the smaller dose (4 hrs. and 3 hrs., respectively). The increased time involved in return of the blood sugar level to the initial base line value with larger amounts of dextrose was reflected in a tendency for the hypoglycemic reactions to be correspondingly delayed.

Considerable variation was found in any one subject in regard to the amplitude of the blood sugar rise and general shape of the tolerance curve after sugar ingestion. These differences were probably due, in part, to the attending nausea which occasionally followed ingestion of the test solution. The wide variation seen in these data do not permit any conclusions regarding differences in the initial peak of the tolerance curve for either ground level or altitude with either dose. Less variation did occur, however, with respect to the time at which the blood sugar level approached the pre-ingestion base line value although even in this regard it was found that at least several tolerance curves were necessary

TABLE 3

*Effect of glucose ingestion of varying dose at ground level and altitude**

SUBJ.	GLUCOSE	TOTAL NO. TESTS		TOTAL NO. REACTIONS		ONSET†		DURATION		SEVERITY‡	
		G.L.	Alt.	G.L.	Alt.	G.L.	Alt.	G.L.	Alt.	G.L.	Alt.
	(gms.)					(hrs.)	(hrs.)	(mins.)	(mins.)		
S. K.	70	3	3	1	1	3.0	4.0	15	30	++	++
	150	1	1	1	1	4.25	5.0	15	§	+	+++
C. K.	70	1	2	1	1	3.5	4.0	45	15	++	++
	150	1	1	1	0	5.25		15		+++	
L. K.	70	3	1	2	1	4.0 4.5	4.25	60 60	60	+++ ++	+++
	150	1	1	1	0	5.0		§		+++	
G. B.	70	2	2	0	2		3.25 4.5		00 5		+++ +
	150	1	1	1	1	4.0	4.5	60	§	+++	+++
R. B.	70	2	2	2	2	4.0 4.25	4.25 3.75	60 20	60 60	+++ +	+++ +++
	150	1	1	1	1	4.5	4.0	45	60	+++	+++
S. D.	70	2	3	0	0	—	—	—	—		
	150	1	1	0	0	—	—	—	—		
Total.....		19	19	11	10	4.20	Av. 4.15	40.5	Av. 43.7		

* The 70 gram dextrose dose was used at 8,000 feet; the 150 gram dose at 10,000 ft.

† "Onset of Reactions" represents within one-quarter of an hour the time (in hours) after ingestion of sugar when the reactions began.

‡ The severity of the hypoglycemic reactions was judged arbitrarily as follows:

"+" = "Mild" Reaction characterized by paresthesia, nervousness, and an empty, hungry feeling. Subject indicated he felt completely better within 15 minutes after onset of reaction.

++ = "Moderate". Subject manifested objective signs of tremors and sweating in addition to subjective symptoms.

+++ = "Severe". Reaction was adjudged severe when there were pallor, marked tremors, profuse sweating, intense hunger and weakness, with the latter two symptoms still persisting at the end of the experiment.

§ Objective reactions still present at completion of experiment.

in the same individual to establish a central tendency. Accordingly, the data on the dextrose tolerance tests are to be considered significant only as reflecting group rather than individual characteristics.

TABLE 4
Mean blood sugar values (mgm. %_o) in glucose tolerance tests

ALT. (GLUCOSE GRAMS)	FASTING BLOOD SUGAR	MEAN BLOOD SUGAR VALUES AFTER GLUCOSE INGESTION (HRS.)						
		0.5	1	1.5	2	3	4	5
G. level (70)	97 \pm 10* (2.8)†	195 \pm 38 (10.6)	194 \pm 47 (12.9)	180 \pm 44 (12.1)	160 \pm 42 (11.7)	101 \pm 33 (9.1)	88 \pm 21 (5.8)	89 \pm 20 (5.7)
8,000 ft. (70)	100 \pm 10 (2.8)	174 \pm 26 (7.9)	172 \pm 33 (9.2)	159 \pm 37 (10.2)	140 \pm 37 (10.5)	96 \pm 20 (5.7)	93 \pm 14 (3.8)	97 \pm 11 (3.2)
G. level (150)	103 \pm 18 (7.3)	180 \pm 24 (9.8)	181 \pm 40 (16.5)	147 \pm 33 (13.6)	148 \pm 16 (6.7)	133 \pm 34 (14.0)	100 \pm 40 (16.2)	85 \pm 31 (12.6)
10,000 ft. (150)	102 \pm 17 (7.0)	197 \pm 25 (10.0)	201 \pm 46 (18.6)	202 \pm 44 (17.9)	192 \pm 33 (13.6)	158 \pm 32 (13.2)	106 \pm 36 (14.5)	99 \pm 18 (7.5)

* Mean Blood Sugar \pm Standard Deviation.

† Standard Error of Mean.

Above values represent the means of 13 tolerance tests at ground level and 13 tests at 8,000 feet with the 70 gram dose, and 6 tests at ground level and 6 tests at 10,000 feet with the 150 gram dose.

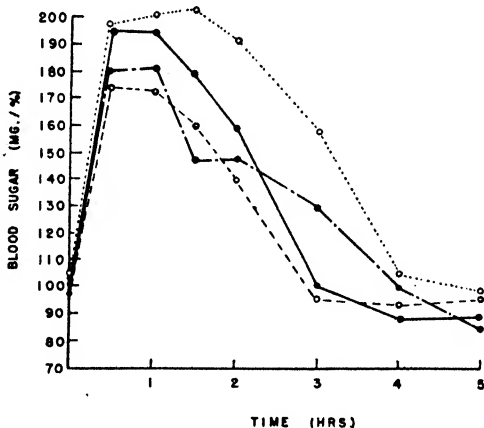


Fig. 2. Comparison of mean tolerance curves, for all subjects combined, at altitude and ground level with 70 and 150 grams of dextrose.

Curves traversing solid circles represent ground level. Solid line refers to average of 13 tests at ground level using the 70 gram dose; the broken line refers to the mean of 6 tests with 150 grams.

Curves traversing hollow circles represent altitude. Broken line refers to average of 13 tests at 8000 ft. using the 70 gram dose; the dotted line, to the mean of 6 tests at 10,000 ft. using the 150 gram dose.

Blood sugar values at 0 time (approximately 5 hrs. after start of observations) were obtained just prior to ingestion of sugar.

From an examination of the mean glucose tolerance curves for all subjects combined, it appears that a difference in the hypoglycemic component of the curve exists between altitude and ground level. This difference is more apparent when the blood sugar values taken from individuals during a hypoglycemic episode are directly compared (table 5). The depression of blood sugar values below the pre-ingestion level is greater at ground level than altitude and this depression tends to persist. The blood sugar level at altitude, one hour after the onset of the reaction, has approached the pre-ingestion level. These data thus indicate that at altitude the hypoglycemic component of the dextrose tolerance curve tends to be shortened. This difference in hypoglycemic "dip",

TABLE 5

Blood sugar values just prior to ingestion of glucose and during hypoglycemic reactions at altitude and ground level

GROUND LEVEL (800 FT.)		BLOOD SUGAR (MG. %)			ALTITUDE (8,000-10,000 FT.)		BLOOD SUGAR (MG. %)		
Pre-ingestion (1)	Onset of reaction (2)	mgm. % (1-2)	1 hr. after onset	mgm. % (1-3)	Pre-ingestion (1)	Onset of reaction (2)	mgm. % (1-2)	1 hr. after onset	mgm. % (1-3)
89	43	-46	52	-37	97	77	-20	83	-14
112	90	-22	90	-22	98	86	-12	96	-2
86	65	-21	44	-42	104	99	-5	92	-12
106	73	-33	87	-19	93	85	-8	77	-16
102	91	-11	88	-14	96	89	-7	92	-4
125	52	-73	70	-55	87	85	-2	100	+13
77	62	-15	36	-41	88	83	-5	98	+10
101	111	+10	115	+14	112	86	-26	87	-25
122	74	-48	—	—	102	81	-21	—	—
95	114	+19	—	—	111	80	-31	98	-13
95	75	-20	83	-12					
* Mean blood sugar values and in parentheses, the standard error of the mean.									
— Experiment completed before one hour elapsed.									
101 (5.2)	77 (6.8)	-23.6	74 (8.5)	-25.3	99 (2.8)	85 (1.9)	-13.7	92 (2.6)	-7.0

* Mean blood sugar values and in parentheses, the standard error of the mean.

— Experiment completed before one hour elapsed.

however, cannot be positively correlated with any difference in severity or duration of the hypoglycemic attack.

Further analysis of the data in table 5 reveals that the development of hypoglycemic reactions occurred at widely varying blood sugar values although in the majority of cases the values were below those of the initial fasting level. There appeared to be less variation at altitude in this regard, however. An appreciable number of reactions took place with blood sugar values at 90 mgm. per cent or over, suggesting that the production of these episodes did not necessarily depend upon the absolute level of the blood sugar "per se". There was some evidence that their development was associated more with the suddenness with which the blood sugar level fell from a pre-existing peak. In those cases where

the blood sugar values in hypoglycemia were relatively high it was found that the individual tolerance curves showed differences in sugar values, between onset of the reaction and the previous determination ($\frac{1}{2}$ -1 hr.), sometimes exceeding 100 mgm. per cent.

DISCUSSION. The confused state of evidence for the effect of anoxia on the blood sugar has recently been summarized and clarified to some extent by Van Liere (6) and Van Middlesworth *et al.* (7). Much of the literature on this point is highly controversial and the need for more well controlled work on animals and man is clearly indicated. Hyperglycemia has been frequently described (8, 9, 10), yet others have indicated no change or even lowering of the blood sugar in anoxia (11, 12). These discrepancies may be partially accounted for by differences in the experimental procedure, such as suddenness and severity of the anoxia, duration of the exposure, acclimatization, and the previous nutritional state. The results of the present experiments show clearly that the blood sugar level in unacclimatized individuals maintained at simulated altitudes of 8,000 and 10,000 feet for prolonged periods, following controlled conditions of food intake, is not significantly different from that at ground level. In either instance, the blood sugar level remains well within accepted limits of normality over the entire exposure period. The similar results obtained by the use of two well accepted blood sugar methods is doubly reassuring in this regard. The present findings confirm, in part, the work of Forbes (13) which demonstrated no substantial change in the blood sugar level of recently acclimatized human beings at various altitudes up to 5,340 meters (17,520 ft.).

The presence of a normal blood sugar level for these prolonged periods at moderately low altitudes has an important bearing on the problem of maintaining well-being and efficiency in flying personnel on protracted flights. King *et al.* (2) have demonstrated that altitude tolerance is consistently poor when exposures are made without a preceding meal and suggest that this may be due to lowered reserves of cellular carbohydrate. Poor performance and fatigue reactions among pilots have been associated with inadequate pre-flight feeding. Although hypoglycemia has been suspected of playing a rôle in these phenomena, blood sugar determinations have not been made under these conditions. The evidence becomes clear as studies on anoxia continue that nervous tissue is particularly susceptible to oxygen deficiency. It is well established, further, that the energy demands for normal function of the brain and related tissues are met almost exclusively from carbohydrate and, thus, are contingent upon a continuous supply of adequate blood sugar (14). Electro-encephalographic studies present more direct evidence of the undesirability of low blood sugar levels at altitude. Case *et al.* (15) have recently shown that abnormal electrical activity is present in the brain of individuals with lowered blood sugars at altitudes of 10,000 ft. and above, and that such activity is removed by the ingestion of food or sugar. It was found in the present experiments that no apparent correlation existed between an individual's blood sugar level and his ability to tolerate the anoxia. The possibility must not be dismissed, however, that the blood sugar values at altitude, though in a range considered normal at ground level, are still not opti-

mal. It would be of great interest to determine whether or not performance at anoxic altitudes can be improved and sustained as a result of a continuously heightened blood sugar level.

The gain in altitude tolerance achieved with high carbohydrate administration has in some cases been sufficiently marked to prompt some investigators to advocate routine carbohydrate feeding to flight personnel (1, 2, 16, 17). The finding in this investigation that the ingestion of pure carbohydrate in amounts as low as 70 grams can cause the development of adverse reactions at altitude definitely negates, in our mind, any gain in "ceiling" which may possibly result from administered carbohydrate under these conditions. The occurrence of such reactions as weakness, intense hunger, and tremor, either mild or severe, may lead to disastrous results in flight and would be especially dangerous should they develop at or about the time of landing. The time factor (3-5 hrs.) in the development of the reactions is important and probably accounts for the fact that hypoglycemic episodes have not appeared in experiments involving relatively short exposures. Although the reactions were induced by ingestion of pure dextrose, it is not considered unlikely that in some individuals reactions may occur after high carbohydrate feeding. Cases of spontaneous hypoglycemia---in which the symptoms resemble those seen in this study---are fairly numerous in the clinical literature, and various investigators have reviewed the evidence which indicates that relatively high carbohydrate meals predispose to hypoglycemia (18, 19). The production of such reactions, however, does not necessarily contra-indicate the use of carbohydrate to gain altitude if precautions are taken to determine the proper intervals of time at which maximal amounts may be safely ingested.

Few reports are available in the literature regarding carbohydrate tolerance at reduced barometric pressure, and these appear to be highly contradictory. Aggazotti (20) described an increased tolerance to sugar, a finding later unconfirmed by Ferraloro (21) in acclimatized human beings at 9,500 ft. Forbes (13), however, reported greatly increased tolerance in two subjects at 5,340 meters. Michaels and Sundstroem (22) obtained data suggestive of an initial decrease, in the rat, followed weeks later by an increase in tolerance. It was very apparent in this study, as others have shown, that dextrose tolerance can be quite variable in any one individual. There was no convincing evidence, however, when several tests were done, that carbohydrate tolerance was significantly altered at the altitudes studied. It was found more expedient because of the practical implications of flight to test tolerance at the midpoint of the exposure period, 4 to 5 hours after the standard meal rather than in the usual clinical manner (12-14 hrs. post-absorptive). Under these conditions a super-imposition effect may have occurred. It is not considered likely, nevertheless, that the results obtained would be appreciably different with the usual method of testing.

There was some evidence from these experiments that the homeostatic mechanism governing the blood sugar level may be influenced by prolonged moderate anoxia. This was suggested by the fact that the hypoglycemic phase (dip below the base line) of the tolerance curve was shortened at altitude. A similar finding

has been reported by Leipert and Kellersman (23) at higher altitudes (18,000–20,340 ft.). In the present work the more sustained depression of the blood sugar at ground level became evident when values during the hypoglycemic episodes were directly compared. The earlier restoration of the blood sugar to the pre-ingestion level at altitude did not appear to reflect any difference in severity of the symptoms. Somogyi and his colleagues (24, 25) have demonstrated both in the dog and in the normal and diabetic human being that hypoglycemia induced either by glucose or insulin is eventually followed (3–6 hrs.) by a compensatory hyperglycemia in which the blood sugar temporarily exceeds the fasting level. It is possible that the earlier rise of the blood sugar level at altitude may represent the beginning of this secondary hyperglycemic rise—a phenomenon apparently delayed at ground level. Until more is known of the delicately regulated balance between glycogenolytic and glycogenetic processes controlling the blood sugar level, further consideration of this change at altitude would be speculative. Additional experiments are needed before the liver or the endocrine glands can be differentially implicated in the phenomenon.

CONCLUSIONS

1. No significant change from the ground level condition occurs in the blood sugar level of resting human subjects maintained at simulated altitudes of 8,000 and 10,000 ft. for periods of 10 hours without supplementary oxygen.

2. The ingestion of pure carbohydrate (dextrose) at these altitudes can cause the development of secondary hypoglycemic reactions similar to those induced at ground level, and sufficiently marked to affect adversely well-being and efficiency in flight.

3. Carbohydrate tolerance, as measured by the amount of time required for the blood sugar level to attain pre-ingestion values, is not appreciably altered in human subjects at moderately low altitudes. There is suggestive evidence, however, that the homeostatic mechanism controlling blood sugar level is affected.

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COMPARISON OF RENAL CLEARANCES WITH DIRECT RENAL BLOOD FLOW UNDER CONTROL CONDITIONS AND FOLLOWING RENAL ISCHEMIA¹

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In a previous report (1) it was demonstrated that the renal plasma clearances of p-amino hippuric acid (PAH) and creatinine were simultaneously reduced in dogs following clamping of the renal artery, the degree and duration of reduction being proportional to the length of the period of ischemia. Such reduction in clearance is indicative of increased renal vascular resistance, possibly resulting from afferent arteriolar constriction, inasmuch as the clearances were proportionately reduced. There occurred, however, simultaneous decrease in the plasma extraction ratio of PAH, and changes in the concentration ratio of creatinine, suggesting that ischemia had impaired the excretory and concentrating ability of the renal tubules. In the present report, therefore, by observing direct renal blood flow concurrently with renal clearances it was possible to evaluate the validity of the clearance of PAH at low plasma concentrations as a measure of renal plasma flow, and the clearance of creatinine as a measure of glomerular filtration rate following a 20 minute period of complete renal ischemia.

Additional data are presented from a total of 17 control experiments in which clearance of PAH at low plasma concentrations, plus hematocrit volume, has been compared with the whole renal blood flow by a direct method. These comparisons are of significance because of the current interest in the use of this method introduced by Smith and his associates (2, 3) for measuring effective renal plasma flow.

EXPERIMENTAL PROCEDURE. Male and female dogs ranging in weight from 10 to 18 kgm., anesthetized with 30-35 mgm./kgm. of body weight of pentobarbital sodium administered intravenously, were used in these experiments. The left kidney was approached by a ventral incision in most experiments; on a few occasions a flank approach was used. The left ureter was cannulated close to the kidney to minimize dead space, and the left renal vein and artery were dissected sufficiently away from surrounding tissue to allow placement of ligatures. Ovarian or spermatic veins were ligated at the point of juncture with the renal vein. Femoral arteries were cannulated for continuous registration of mean blood pressure and for removal of arterial blood for analysis of infused substances; one femoral vein was cannulated for infusion. Both external jugular veins were prepared for cannulation.

Direct blood flow through the left kidney was measured by a modification of a method used by Blalock and Mason (4). The cannula employed was con-

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structed from thin-walled brass tubing of 0.5 cm. inside diameter. It was 46.5 cm. long, slightly bent at the tip, which contained several large perforations. A small elevated collar about 1 cm. from the end insured secure ligation. The cannula was introduced into the right external jugular vein with a slow stream of saline flowing through it, and was gently passed down into the abdominal vena cava so that the tip lay opposite the left renal vein as visualized through the ventral incision. The animal was then heparinized with 0.4 cc. per kgm., with additional amounts during the course of the experiment (0.5 cc. every half-hour).² The cannula was then introduced into the renal vein and securely ligated. Such a cannulation procedure does not require interruption of the renal blood flow. This is an important feature of the method, for it has been shown that even brief cessation of renal flow may influence the renal clearances (1). Renal vein blood was then allowed to flow from the renal cannula, projecting to the exterior from the right jugular vein, through a direct circuit back into the opposite (left) jugular vein. A shunting key was arranged so that, when depressed, the return circuit to the opposite jugular vein was occluded while simultaneously opening a shunt circuit allowing the renal vein blood to flow into 10 cc. graduated cylinder. Depression of the key also actuated, by means of a mercury contact, an electric chronometer calibrated to 1/100's of a second, thus timing accurately the period of outflow. From this, renal blood flow in cc./min. was calculated. The outflow cannula of the shunt circuit was placed at a height equal to the pressure in the renal vein circuit, as indicated by the level of blood in a reservoir through which the renal vein blood aliquots were immediately reinfused. This reservoir was connected to a T-cannula common to the renal vein circuit and the left external jugular vein.

The resistance offered by the renal cannula to blood flow was tested by observing the differential pressure across the cannula at different rates of blood flow. This resistance was found to be equivalent to 3.5 mm. Hg at 100 cc. per minute. Such a pressure loss is thought to be of about the same order of magnitude as the pressure difference between the abdominal vena cava at the point where the kidney *in situ* drains and the pressure in the external jugular vein at the point where cannulated renal vein blood was reinfused. It is concluded that this method measured renal blood flow correctly at the rates of flow realized.

Usually about an hour elapsed between the completion of the surgical procedures and the beginning of the renal blood flow and clearance determinations. During this time interval the above renal cannulation was performed, and an average volume of 22 cc./kgm. of body weight of warm saline was infused to provide an adequate urine flow for clearance determinations. Priming doses of 0.25 gram of creatinine and 50–100 mgm. of PAH³ were followed by continuous infusion of 0.9 per cent saline containing 0.8 per cent creatinine and 0.09 per cent PAH at the rate of 1.3 cc./min. In a few experiments the infusion fluid contained

² The heparin used was either "Liquaemin," supplied by Roche-Organon, Inc., Nutley, N. J., or the Connaught Laboratories preparation. One cubic centimeter of solution contained 10 mgm. of heparin.

³ We are indebted to Dr. Karl H. Beyer of Sharpe and Dohme, Inc., Glenolden, Pa., for the supply of p-aminohippuric acid used in these studies.

12.5 per cent mannitol, used as an osmotic diuretic; with this a 5 gram priming dose of mannitol was given. Following two control urine collection periods of approximately 15 minutes each, a clamp was applied to the renal artery, completely occluding it for 20 minutes. After release of the clamp, urine collection was resumed after an average interval of 17 minutes. Direct blood flow determinations were simultaneously made at intervals averaging approximately 2 minutes throughout the course of the experiment.

To determine clearances, creatinine and PAH concentrations obtained from frequent arterial samples, usually one per urine collection period, were interpolated to the midpoint of each urine period corrected for emptying time. The emptying time was approximated from an estimation of the renal dead space and the rate of urine flow. Plasma proteins were precipitated by the CdSO_4 method (5). Creatinine was analyzed in a photoelectric colorimeter by the alkaline picrate method (6) and PAH was analyzed by Smith's modification (3) of the method of Bratton and Marshall (7).

Hematocrit determinations were made by spinning blood in Wintrobe hematocrit tubes for $\frac{1}{2}$ hour at 2,000 R.P.M. (Tubes spun an additional 30 minutes at 3,000 R.P.M. decreased in reading by only 1 cell volume per cent indicating that the shorter centrifugation had been adequate for complete packing of the red blood cells.) A correction factor of 0.96 was applied for the plasma trapped between cells (Gregersen and Schiro (8)).

RESULTS. *A. Comparison of direct blood flow with blood flow derived from renal clearances under control conditions.* A total of 17 experiments averaging 33 minutes in duration has been performed in which the renal blood flow (BF_{pah}) calculated from the renal clearance of PAH and hematocrit volume has been compared with the whole blood flow (WBF) determined by the above direct method (table 1). The results show that the ratio $\text{BF}_{\text{pah}}/\text{WBF}$ averages 0.91 (S.D., 0.113).⁴ Inasmuch as the average difference between mean BF_{pah} and WBF of all experiments falls within the limits of the standard deviation, it is our opinion that there is no significant difference between renal blood flow based on the clearance of PAH and direct blood flow. It is possible, however, that more refined techniques for correlating direct blood flow and clearance may show that there is, in fact, an "effective" renal plasma flow somewhat less than total plasma flow, as has been suggested by Smith (9) and is indicated by the PAH extraction ratio of 0.87 found by Phillips *et al.* in dogs (10).

⁴ A possible technical source of variation between these two types of measurement should here be mentioned and is concerned with the correlation of the period of urine collection with the period of WBF measurement, since this involves a lag between the time the urine is formed at the glomeruli and the time it is collected. This probable source of error is considered of consequence only when the urine flow is very low, or when there are rapid changes in direct blood flow. An "emptying" correction factor has nevertheless been applied, based on an estimation of the dead space in the collection system and the rate of urine flow. However, in 12 of the 17 experiments, the rate of urine flow was in excess of 0.5 cc. per minute per kidney, requiring minor corrections, and the remaining experiments with lower rates of urine flow are in good agreement with these. A similar correction was made for the periods following renal ischemia.

TABLE 1

Control data comparing whole blood flow with simultaneous *p*-aminohippuric acid clearance

			p-AMINOHIPPURIC ACID CLEARANCE*				WHOLE BLOOD FLOW†				Av. MBP mm. Hg
Expt. no.	Wt. kgm.	Urine vol. cc./min. per kidney	Plasma mgm. per cent	Uv/p cc./min. per kidney	Uv/p cc./min. per kidney sq.m. S.A.†	BF _{pah} § cc./min. per kidney per sq.m. S.A.	ER _{pah}	cc./min. per kidney	cc./min. per sq.m. S.A.	BF _{pah} WBF	
A. Mannitol diuretic											
1	13.5	1.00	1.00	65.0	102.0	182.0	0.670	130.0	203.0	0.90	138
2	10.5	1.31	0.65	72.3	135.0	235.0	0.701	137.5	257.0	0.91	127
3	13.0	1.35	0.76	71.5	115.5	248.0	0.735	162.0	261.0	0.95	131
Av....		1.22	0.80	70.0	117.0	222.0	0.702	143.0	240.0	0.92	132
B. Saline diuretic: 14.4 cc./kgm. (range, 7-20 cc./kgm.)											
4	18.0	0.73	0.22	147	194	422	0.935	319	420	1.000	149
5	12.0	0.30	0.714	104	178	301	0.800	193	330	0.913	130
6	14.0	0.65	0.785	82	125	240	0.703	174	264	0.910	134
7	10.0	0.20	0.790	85	163	255	0.635	125	240	1.06	132
8	16.0	0.320	0.83	75.5	106	212	0.653	171	240	0.885	134
9	13.0	0.290	0.85	98.5	159	256	0.715	168	271	0.945	137
10	14.5	0.200	0.87	73	110	183	0.715	135	203	0.900	130
11	14.0	0.580	0.91	59	91	164	0.670	122	188	0.87	138
Av....		0.41	0.75	91	140	252	0.735	176	270	0.935	135
C. Saline diuretic: 33 cc./kgm. (range, 21-43.5 cc./kgm.)											
12	14.0	0.85	0.45	140.0	215.0	347	0.765	291	448	0.775	119
13	13.0	0.62	0.48	119.5	193.3	282	0.853	173	280	1.01	82
14	10.0	0.67	0.52	136.0	262.0	430	0.800	209	403	1.07	139
15	11.0	0.72	0.95	46.0	84.0	154	0.760	137	249	0.62	111
16	15.0	0.77	1.12	86.0	127.0	212	0.800	144	213	0.99	120
17	11.5	0.80	1.82	123.0	216	343	0.640	258	453	0.76	121
Av....		0.74	0.89	109.00	183	296	0.770	203	340	0.870	116

* All clearance data represent the average of two consecutive urine collection periods, with the exception of experiments 2 and 3, where 4 consecutive urine collection periods, totaling approximately an hour, are averaged. The control data of table 2 are included in this table.

† Whole blood flow (WBF) observations were made at intervals averaging approximately 2 minutes. Average duration of each experiment is 33 minutes.

‡ Surface area (S.A.) was determined according to the Meeh-Rubner equation:

$$\text{surface area in square meters} = \frac{11.2 \times W^{2/3}}{100}$$

where W is body weight in kilogram.

§ BF_{pah} = renal blood flow derived from plasma clearance of PAH plus hematocrit volume.

|| ER_{pah} = plasma extraction ratio of PAH: $\frac{A - V}{A}$, where A and V represent the arterial and renal venous plasma concentrations in milligram per cent. Usually two determinations per experiment are averaged into this column.

It can be noted that there is a correlation between the amount of saline given and the rate of renal blood flow and renal clearance. Larger amounts of saline (part C) have a hyperemic effect, probably a result of increase in circulating blood volume.

The possible influence of renal cannula resistance on renal clearance can be evaluated by comparing the data of table 1 with data obtained from dogs with uncannulated renal veins. The average of all control PAH clearances in the present series of anesthetized, operated dogs with cannulated kidneys was 151 cc./min. per kidney/sq.m. S.A. (S.D. 51). This compares favorably with another series of 64 observations (right and left kidneys) in anesthetized, operated dogs, differing only in that the renal veins were not cannulated (1). The average of the PAH clearances in the latter series was 141 cc./min. per kidney/sq.m. S.A. (S.D. 46), indicating that the influence of the renal cannula on renal blood flow was negligible.

It is also important to compare renal clearance of the present series with renal function in unanesthetized dogs. These observations on anesthetized, operated dogs are in agreement with the data derived by Houck and his co-workers (11) from 203 observations on 52 normal, unanesthetized female dogs where the PAH clearance for both kidneys averaged 290 cc./min. sq.m. S.A. (S.D. 64). It is of further interest to compare the present whole renal blood flow measurements with the data of Mason, Blalock, and Harrison (12) who employed a somewhat similar cannulation technique for direct blood flow measurement in unanesthetized dogs. They report an average whole blood flow of 10.5 cc./min. per kidney per kgm. of body weight (range, 8–14.9 cc./min.), while the data of this report average 13.8 cc./min. per kidney per kgm. of body weight (range, 8.7–22.4). Since these comparisons are reasonably consistent, the opinion is held that the present series of anesthetized, operated dogs with cannulated renal veins may be validly used to compare direct renal blood flow with blood flow derived from the clearance of PAH under control conditions.

B. Effect of complete ischemia on direct renal blood flow and on mean blood pressure. A total of five experiments were performed in which renal clearances and WBF were compared following a 20 minute period of complete renal ischemia. Four of these experiments are summarized in table 2 and two representative experiments appear in figure 1. A fifth experiment was omitted from consideration because the control mean blood pressure was somewhat low (av., 82 mm. Hg), and fell to lower levels after the release of the clamp (av., 71 mm. Hg). This hypotension may have contributed to the anuria of over one hour's duration which followed the release of the clamp in this dog, thus making it impossible to compare renal clearances and WBF during this interval.

In the experiments of table 2, observations on WBF have been divided into four periods to coincide with the intervals of urine collection. The first of these urine collection periods was a discard period, and so no comparison of clearance and WBF appears in the table for this period. The first period of WBF observation averaged 17 minutes in length, following the release of the arterial clamp, and WBF averaged 41 per cent of the control mean (range 30 to 52 per cent). After an average interval of 37 minutes the second WBF averaged 40 per cent of the control mean (range 21 to 50 per cent). At 58 minutes the average of the third experimental period was 46 per cent of the control mean (range 33 to 56 per cent), and the fourth period (85 min.) averaged 55 per cent of the control (range 47 to 72 per cent).

In three of the four experiments (nos. 2, 3, and 4) included in table 2, there was no significant change in mean blood pressure during the course of the experiment (ratio of MBP following the clamping to the control, 0.97, 0.82 and 1.03 respectively). In the remaining experiment (no. 1), the ratio fell to 0.73, but the mean blood pressure remained high (87 mm. Hg) and the results are considered comparable to the other three experiments. As a matter of fact, this experiment

TABLE 2

The effect of 20 minutes of ischemia on whole renal blood flow and renal clearances

EXPT. NO.	TIME IN MIN. AFTER RELEASE OF CLAMP	WHOLE BLOOD FLOW		MBP mm. Hg	URINE CC./MIN. PER KIDNEY	D-AMINOHIPPURIC ACID CLEARANCE							CREATININE CLEARANCE		
		cc./min. per kidney	Expt./control			Plasma mgm. per cent	Uv/p cc./min. per kidney	Hematocrit per cent	BF _{pah} cc./min. per kidney	Expt./control	BF _{pah} WBF	ER _{pah}	Uv/p cc. min. per kidney	Expt. control	U p
1 (15 kgm.)	Control*	144.0		120.0	0.77	1.12	89.3	40.5	150.0		1.04	0.800	23.5		38.0
	4-17	60.0	0.417	89.0											
	17-31	71.0	0.493	87.0	0.07	1.40	2.4	42.0	4.1	0.027	0.058	0.550	0.7	0.03	10.0
	31-48	81.0	0.563	85.0	0.06	1.55	1.6	42.0	2.8	0.019	0.034	0.612	0.5	0.02	8.4
	48-73	104.0	0.723	88.0	0.56	1.64	75.5	39.0	124.0	0.830	1.19	0.720	15.5	0.66	27.6
2 (11.5 kgm.)	Control	258.0		121.0	0.80	1.82	123.0	37.0	195.0		0.757	0.640	42.4		53.6
	2-17	77.0	0.300	125.0											
	17-34.5	54.2	0.210	120.0	0.06	2.00	9.0	40.0	15.0	0.077	0.278	0.347	3.0	0.071	49.0
	34.5-58	86.3	0.334	111.0	0.08	2.06	13.0	39.0	21.0	0.108	0.244	0.420	5.4	0.127	63.5
	58-86.5	122.0	0.473	110.0	0.19	2.00	39.0	38.0	63.0	0.323	0.517	0.378	11.5	0.271	59.5
3 (11 kgm.)	Control	137.0		111.0	0.716	0.950	46.0	45.5	84.5		0.62	0.762	9.1		12.8
	1-14	71.0	0.520	86.0											
	14-41	68.0	0.496	89.0	0.073	1.50	8.7	43.0	15.0	0.177	0.22	0.610	1.07	0.118	14.7
	41-61	66.0	0.480	95.0	0.125	1.23	44.0	40.0	73.5	0.870	1.11	0.615	7.50	0.825	60.0
	61-86	67.5	0.493	92.0	0.120	1.33	33.0	40.0	55.0	0.650	0.815	0.705	6.20	0.680	51.5
4 (14 kgm.)	Control	291.0		119.0	0.853	0.45	140.0	38.0	226.0		0.770	0.765	35.00		41.3
	1-20	117.0	0.400	131.0											
	20-43	119.0	0.410	121.0	0.208	0.82	45.0	42.0	78.0	0.345	0.655	0.655	10.0	0.286	47.0
	43-64	134.0	0.460	120.0	0.262	0.80	57.5	41.0	97.5	0.430	0.730	0.620	13.0	0.372	50.0
	64-94	152.0	0.522	116.0	0.350	0.73	71.0	41.0	120.0	0.530	0.790	0.620	15.6	0.445	45.0

* Two periods are averaged into the control data.

For interpretation of abbreviations, see footnotes of table 1.

showed the greatest degree of recovery of WBF (to 72 per cent of the control mean at the end of the experiment).

C. Effect of ischemia on renal clearance and the rate of urine flow. The renal plasma clearance of PAH was more markedly influenced by the 20 minute period of complete ischemia than was the simultaneous WBF. Consequently, during periods comparable to the later three periods of WBF observation (the first being a urine discard period), BF_{pah} averaged 15.6, 35.7, and 58.3 per cent of the mean control values. (For range of values, see table 2, column 11, headed "Exp./cont." for PAH clearance.)

The reduction in creatinine clearance closely paralleled the changes in BF_{pah} , during the same periods of observation averaging 12.6, 33.6 and 51.4 per cent (column 15, table 2) of the control mean, respectively. During these periods urine flow declined to 13, 17 and 39 per cent of the average control flow. Figure 1 graphically portrays the difference in effect of ischemia on WBF, BF_{pah} , and creatinine clearance in two experiments, no. 1 and no. 2 of table 2.

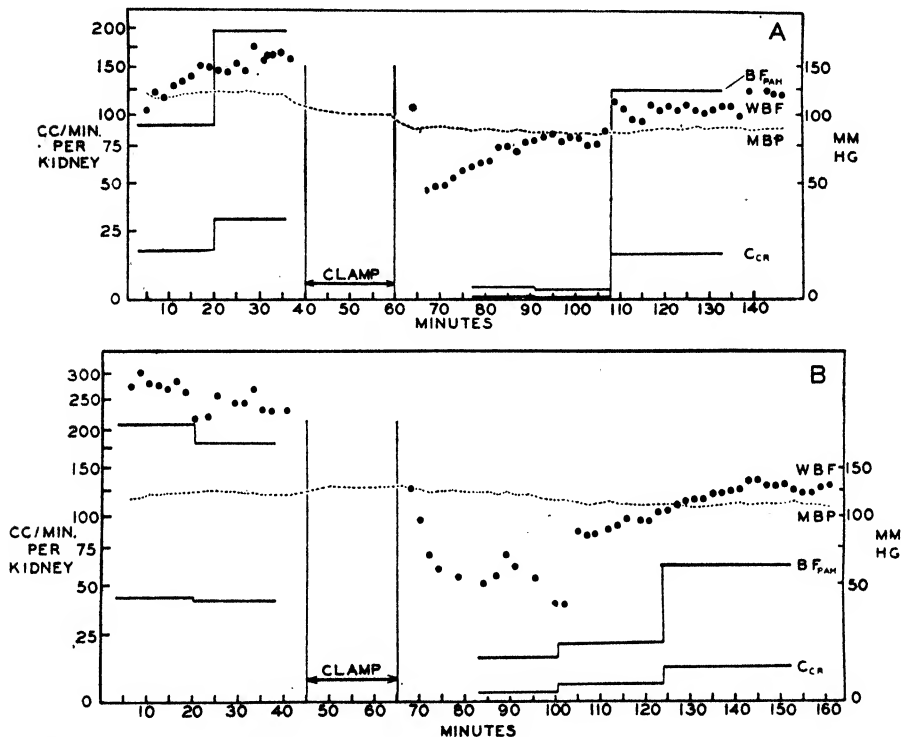


Fig. 1. Showing the effect of complete renal ischemia on renal blood flow and renal clearances in two representative experiments (no. 1 and no. 2, table 2). WBF: (solid circles) whole blood flow determined by the direct method. BF_{pah} : (upper solid line) renal blood flow derived from the plasma clearance of PAH plus hematocrit volume. C_{cr} : (lower solid line) creatinine clearance. MBP: (dotted line) mean systemic blood pressure.

D. Effect of ischemia on the plasma extraction ratio of PAH and the concentration ratio of creatinine. It may be noted in table 1 that the average control plasma extraction ratio of PAH (ER_{pah}) is 0.74 (range, 0.64 to 0.935) at plasma concentrations ranging from 0.22 to 1.82 mgm./per cent. Since the ratio C_{pah}/ER_{pah} would give an erroneous value higher than the direct plasma flow, and since $BF_{pah}/WBF = 0.91$, it is obvious that the efficiency of tubular excretion of PAH is greater than the apparent incompleteness of the plasma extraction in these experiments would suggest. This could result from contribution of PAH

by the RBCs (3), (10) to the renal vein plasma after withdrawal from the renal vein. It has been pointed out (10) that unless renal vein blood is immediately centrifuged there is opportunity for diffusion of PAH from the RBCs to occur. Immediate centrifugation has not been technically possible here. Since ample time probably elapsed for diffusion of PAH out of the cells and equilibration to occur, it is conceivable that these extraction ratios are more nearly equivalent to whole blood extraction ratios. In fact, Phillips (13) reports approximately 0.74 for whole blood PAH extraction, a figure equal to our average control values. It is believed that similar treatment of all renal vein samples allows at least a qualitative or directional interpretation of the data on extraction ratios.

In each of the four experiments (table 2) in which the arterial clamp was applied the extraction ratio of PAH was depressed following the period of ischemia, although not as markedly as BF_{pah} . Thus, the control average was 0.742 (0.64–0.80), and the average of the first observation following the release of the renal arterial clamp was 0.560 (0.347–0.690). It should be noted that during the first clearance period, when the effects are most marked, WBF averaged only 40 per cent of the control value, and accordingly that the load to the excretory mechanism (calculated from cc. of direct renal plasma flow/sq.m. S.A. x plasma PAH) decreased from an average of 2.30 mgm. per minute per kidney to 1.2 mgm. per minute per kidney. In effect, the tubules exhibit impaired excretory ability in spite of reduced loads to the mechanism.

Control values of the creatinine concentration ratio (U/P) in four experiments averaged 36.4 at an average urine flow of 0.785 cc./min. per kidney (table 2). Following the ischemia, U/P averaged 30 in four experiments in the first period of observation. The urine flow had decreased in these to 0.10 cc./min. per kidney, indicating a loss of concentrating power on the part of the renal tubules, for at the latter urine flow the U/P control kidneys of another larger series of dogs (1) averaged approximately 350.

DISCUSSION. The present experiments on direct renal blood flow confirm the earlier observations based on renal clearances (1), that increased renal resistance follows brief periods of complete renal ischemia. Thus, during time intervals extending to 94 minutes after the release of the arterial clamp, the whole blood flow averaged 46 per cent of the control flow for all observations, with indication of gradual recovery. As was postulated in the earlier report, the effect on simultaneous renal clearances was greater, and these discrepancies were most marked soon after the release of the clamp. Thus, during the first period of comparison extending to an average of 37 minutes after the release of the clamp, the ratio BF_{pah}/WBF averaged 0.30, at 58 minutes 0.53, and at 85 minutes 0.83 (column 12, table 2). This indicated a recovery from the factors which operated to reduce BF_{pah} below WBF even while reduced blood flow as measured by the direct method persisted in the kidney.

The deficit in renal clearances, it is believed, resulted from the action of ischemia on the renal tubules. This is evidenced by the decreased plasma extraction ratio of PAH, indicating impairment of the excretory ability of the tubules, and decreased U/P for creatinine at low urine flow, signifying loss of concentrat-

ing ability of the tubular cells.⁵ These changes have been more fully discussed in the preliminary report (1), and confirm the findings of Van Slyke and co-workers (15) who clamped the renal artery of dogs for periods extending from 2 to 4 hours. They found that after release of the arterial clamp the blood flow may be partially restored to 50 per cent or more of the control rate, but that abnormally small proportions of PAH, creatinine, and urea are extracted from the renal blood. Clearances remain low, and fatal uremia may develop after 4 hours of clamping. Recovery apparently may follow a 2-3 hour period of clamping.

Increased resistance of the afferent arterioles, causing decrease in the effective glomerular filtration pressure to the point where urine formation and renal clearance ceases, could explain the influence of the ischemia on the renal clearance. However, it is significant that especially in experiment 3, table 2, while whole blood flow remained constant at approximately 50 per cent of the control value, indicating a constant degree of arteriolar resistance, there occurred considerable improvement in clearance, e.g., from 17.7 to 87 per cent of the control value for PAH, and from 12 to 82.5 per cent for creatinine from the first to the second clearance period after renal clamping. This indicates that reduced glomerular filtration pressure cannot be the only factor operating to produce the reduced clearances.

In the absence of significant changes in hematocrit following the period of ischemia, signifying lack of marked changes in blood viscosity, and in the absence of edema as determined histologically (1), it is tentatively concluded that the increased renal vascular resistance following the period of renal ischemia is largely the result of renal arteriolar vasoconstriction. Much experimental evidence has been brought forward to show that the ischemic kidney is the site of production of pressor substances or their precursors (16). It has been postulated that a substance "renin" is formed here which interacts with a component of the blood plasma, "renin activator" (hypertensinogen, prehypertensin) to form a constrictor substance, "hypertensin" (angiotonin). That this mechanism may be operative in producing renal vasoconstriction in the above experiments is supported by the fact that maximal renal vasoconstriction does not occur immediately upon release of the arterial clamp (see expt. B, fig. 1). This suggests the possibility that a component formed in the kidney must pass into the systemic circulation, there to form a pressor substance which now causes renal vasoconstriction when returned to the kidney. It has been demonstrated that 12 minutes of complete renal ischemia is adequate to cause the appearance of measurable quantities of renin in the renal veins of some dogs and humans (17).

The absence of vasoconstriction in the opposite control kidney (1) and the lack of significant change in systemic MBP suggests that the vascular system of the kidney which has been made ischemic is particularly responsive to the pressor substance. This implies that elements responsible for neurogenic control of

⁵ Shannon and Winton (14) have shown that creatinine appears to be absorbed by the tubules of isolated dog kidneys at urine flows in which there are simultaneously high U/P ratios of inulin. These changes may have resulted from the ischemia to which these kidneys were subjected during transfer from normal circulation to perfusion.

vasoconstriction have been sensitized by the period of ischemia. Schroeder and Steele (18) have demonstrated a progressive increase in sensitivity of the renal vascular bed to the vasoconstrictor action of adrenalin as the result of anoxemia created by clamping the renal artery. Doses which had little effect on the pre-clamped kidney produced intense vasoconstriction in the kidney which had been made ischemic.

SUMMARY AND CONCLUSIONS

Renal blood flow calculated from the renal plasma clearance of p-amino-hippuric acid and hematocrit volume averages 91 per cent of whole renal blood flow measured simultaneously by a direct method in anesthetized dogs (S.D., 11.3 per cent). This confirms the postulate that the clearance of p-amino-hippuric acid at low plasma concentrations is a reasonably accurate measure of renal plasma flow under control conditions.

Following a twenty minute period of complete renal ischemia, there occurs a prolonged period of increased renal vascular resistance, evidenced by a reduction in the volume of direct blood flow. The final periods of observation, after an average interval of 85 minutes following the release of the arterial clamp, average 55 per cent of the control mean. No significant change in systemic mean blood pressure occurred which could be related to the changes in renal blood flow. It is suggested that the increased renal vascular resistance is largely the result of arteriolar vasoconstriction.

Renal tubular impairment results from the anoxemia created by renal arterial occlusion, as evidenced by reduction in the plasma extraction ratio of p-amino-hippuric acid and by decrease in the concentration ratio (U/P) of creatinine at reduced urine flows. These changes are reflected in a greater percentile reduction in the clearances of p-aminohippuric acid and creatinine than the simultaneous direct blood flow, resulting in discrepancies of renal blood flow based on the clearance of p-aminohippuric acid. This indicates that renal vascular studies based on clearances cannot be relied upon for quantitative estimates under conditions where the kidney is subjected to complete or severe ischemia and related anoxia. A factor contributing to the disparity between direct blood flow and the renal clearances may be a reduction in glomerular filtration pressure, created by the arteriolar vasoconstriction which follows the period of ischemia.

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FURTHER OBSERVATIONS ON INDUCED OVULATION IN THE MOUSE AS A RAPID TEST FOR PREGNANCY

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It has been shown in earlier reports (Burdick and Whitney, 1941; Burdick et al., 1943) that certain commercial gonadotrophins induced ovulation in the albino mouse within 18 hours after a single subcutaneous injection. It was also reported that mice similarly responded after an injection of 0.5 cc. untreated pregnancy urine.

In the present study further observations have been made on the potencies of pregnancy urines in relation to the duration of gestation.

PROCEDURE. Samples of morning urine were placed in the refrigerator as soon as received. No preservative was added. The urines were injected into the mice with no attention being given to temperature of the specimen. In some instances no attempt was made to make a critical study of minimal ovulating doses of the urine while certain urines had to be diluted at the time of injection to determine minimal ovulating doses. A few specimens from later pregnancies were concentrated by evaporation at room temperature.

Mature albino diestrous mice of the Rockland strain weighing at least 20 grams were selected by the vaginal smear method. Only those showing an abundance of leucocytes typical of diestrus were used. A few late immature mice weighing more than 14 grams and some pregnant mice were injected for comparison. All mice received only one subcutaneous injection and the autopsies were usually performed 18 to 24 hours later. As we have indicated in earlier reports, the positive reaction of the diestrous mouse to chorionic gonadotrophin is ovulation. Evidence of this is easily seen when the reproductive tracts are examined under a dissecting microscope. The ampulla of the oviduct is distended with fluid and the egg mass. The accompanying figures show typical distended ampullae. Figure 2 shows an ampulla which had been flattened under a cover glass so that the eggs could be counted (Burdick and Whitney, 1941). This procedure is necessary only when making quantitative studies for determining threshold doses. The duration of each pregnancy discussed in this report is dated from the first day of the patient's last menstrual period.

RESULTS AND COMMENTS. There was no opportunity to study any urines during the first 4 weeks of gestation, but fortunately we have a rather complete record of the urine of one pregnancy beginning at the end of the first month.¹ At 4 weeks the minimal ovulating dose was 0.5 cc; 0.1 cc. at 6 weeks, 0.05 cc. at 8 weeks, 0.3 cc. at both 10 and 12 weeks and 1.0 cc. at 14 weeks. Expressed in ovulating units per liter of urine, this shows that there were present at 4

¹ These quantitative determinations were made in the laboratory by my former assistant, Ruth T. Rogers.

weeks 2,000 units, at 6 weeks 10,000 units, at 8 weeks 20,000 units followed by a reduction to 1,000 units at 14 weeks. At 16 weeks the ovulating capacity of the urine was greatly reduced as concentrated urine equivalent to 2 or 3 cc. of the fresh material did not cause ovulation. Although such a marked reduction in potency is unusual in our cases, this record agrees in general outline with Browne and Venning's (1936) summary. These workers found a maximum concentration of gonadotrophic substances in urine between the 50th and 60th days and a marked reduction by 120 days after the beginning of the last menstrual period.

Mrs. A. B. submitted a sample of urine when she was approximately 8 weeks pregnant. Ovulation was induced in diestrous mice with 0.05 cc., but not with



Fig. 1



Fig. 2

Fig. 1. Photomicrographs by reflected light of a reproductive tract of a mouse showing the ovary, *O*, uterus, *U*, and distended ampulla, *A*, of the fallopian tube. *T*. Eggs and granulosa cells can be distinguished within the ampulla. The ampulla in a negative pregnancy test has approximately the same diameter as other parts of the oviduct. About 12.5 \times .

Fig. 2. Separate ova by transmitted light after pressure has flattened the ampulla. This procedure is used only for quantitative tests. About 20 \times .

0.03 cc. If 0.05 cc. approximated the minimal ovulating dose, then this urine contained 20,000 mouse ovulating units per liter. Later, at 11 weeks, the potency had decreased so that 0.1 cc. was required to induce ovulation; and at 18 weeks neither 1.2 cc. nor 1.5 cc. was capable of inducing ovulation. This follows the general expectation of reduced potency in the fourth month of pregnancy. Urine from another individual, Mrs. C. S., contained 25,000 units per liter (0.04 cc., minimal ovulating dose) at 3 months but only 5,000 units (0.2 cc., minimal ovulating dose) at 5 months. The gonadotrophic substance from Mrs. C. D. which had 80,000 units per liter (0.0125 cc., minimal ovulating dose) at 8½ weeks dropped to 20,000 units per liter (0.05 cc.) at 11 weeks. This reduction, however is not always so marked as in the case of Mrs. J. S. in which

ovulation was induced with 0.1 cc. at 2 months and yet this response could be brought about with only 0.2 cc. at 8 months. Differences in fluid intake may account for some of these variations.

The history of the pregnancy of Mrs. B. C. is of particular interest. When the urine was first tested the pregnancy had progressed for approximately 4 months. The urine of sp. gr. 1.019 had an unusually high potency at 4 months as only 0.025 cc. urine equivalent was required to induce ovulation, indicating a total of 40,000 ovulating units per liter of urine. When in December male twins were born, we were again reminded of the high titre of the urine in the fourth month. The question naturally arises whether the quantity of chorionic gonadotrophin is consistently greater in the presence of twins than in the presence of a single fetus.

Age of specimen in relation to potency. One specimen of urine which was stored in the refrigerator without a preservative was studied over a period of 30 days. When fresh, only 0.1 cc. was necessary to induce ovulation, but there was a definite reduction in potency 30 days later when 0.3 cc. of the same specimen failed to induce ovulation in diestrous mice and in one animal only 2 follicles ruptured after an injection of 0.4 cc. This agrees with Jares' (1932) report of a reduced potency after 30 days.

Comparison of responsiveness of diestrous and pregnant mice. Ovulation was readily induced in the mated mouse provided the injections were not given within the first three days while the fertilized ova of the pregnant mouse were located in the oviducts (Burdick and Ciampa, 1944). Pregnant animals proved again to be slightly more sensitive to the chorionic gonadotrophin than the mature diestrous mice. With one specimen of urine (Mrs. R. E. at 8 wks.) 0.05 cc. caused ovulation in a pregnant mouse, whereas diestrous mice did not ovulate with less than 0.1 cc. In another case only 0.9 cc. was required to induce ovulation in pregnant mice but 1.0 cc. was the minimal ovulating dose in diestrous animals. This agrees with the results obtained when using a commercial extract of pregnancy urine (Burdick et al., 1943).

Times of ovulation. A few mice were injected with 0.5 cc. of urine to check our earlier observations on the time required for the response of the ovary and the descent of ova into the ampullae. In the earlier experiments with a commercial gonadotrophin, a few instances of ovulation at 16 hours had been seen. Of the 5 mice injected, 2 had not ovulated at 13 and 16 hours respectively, while in the other 3 animals 9 to 10 ova were in the ampullae after 17 to 18 hours. Presumably the period required for this ovarian response may vary in different mice but it is now thought to be completed within 18 hours in diestrous mice.

Superovulation. In a previous report (Burdick et al., 1943) superovulation was recorded in several instances following the injection of pregnant mare's serum, but not in mice treated with an extract of human pregnancy urine. In this present study, superovulation was recorded three times (15, 17 and 21 ova) with the urine of Mrs. B. C. (twins) at 11 weeks. Other instances of superovulation occurred with 8 weeks' urine (Mrs. A. B.) when 17 ova were found after 0.2 cc. and 16 ova in an animal treated with 0.5 cc. urine from a pregnancy of 6 months' duration (Mrs. J. S.).

DISCUSSION. Although an extended critical bioassay of untreated pregnancy urine was not undertaken in this study, the fact has been established that both the mature diestrous mouse and the pregnant mouse respond readily to adequate subcutaneous injections of the urine. It was found in several instances that urines of pregnancies of 6 to 8 weeks' duration contained 10,000 to 80,000 mouse ovulating units per liter.

The amounts of chorionic gonadotrophin, expressed here in terms of mouse ovulating units (M. O. U.), for various intervals during pregnancy are as yet to be considered approximate. Only when 24-hour specimens of urine are analyzed will we have adequate statistical data. Single morning samples are subject to obvious variations. As was pointed out in the case of twinning, sharp deviations from the average may be of clinical significance. It is also possible that this test may be of value in determining such pathological conditions as chorionic epithelioma. It would further appear that ovulation in the diestrous mouse can be used in critical analyses of the ovulating content of various gonadotrophins.

There appear to be certain advantages in this test for pregnancy. The end-point is definite as shown by the swollen ampulla 18 to 24 hours after injection. No histological studies are necessary. These single subcutaneous injections are easily and quickly done in the diestrous mouse which has a wide tolerance to the urine. Only small quantities of urine are necessary and this can be used without extraction or warming. Either the diestrous or pregnant mouse may be used as neither ovulates spontaneously during these periods. Because mice can be kept in a small space at room temperature and be fed cheaply, it is relatively easy to keep a supply on hand. Periods of isolation or preliminary laparotomies are unnecessary and the animals may be used as soon as purchased provided that they are diestrous animals of adequate age and weight or are pregnant.

SUMMARY

Single subcutaneous injections of untreated pregnancy urine cause ovulation in both the diestrous and pregnant mouse. The ampulla of the oviduct becomes distended with fluid and eggs within 18 hours after the injection. This makes a very definite end-point for the test.

Data presented show that the chorionic gonadotrophin content of the urines examined contained 10,000 to 80,000 mouse ovulating units (M.O.U.) per liter of urine from pregnancies of 6 to 8 weeks' duration. There was usually a marked reduction in ovulating capacity by the third month. The potency of stored urine was maintained for approximately 30 days. Several instances of super-ovulation were recorded.

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THE ENERGY COST OF HORIZONTAL AND GRADE WALKING ON THE MOTOR-DRIVEN TREADMILL¹

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Interest in physical fitness is stimulating an increasing amount of research on human work capacity and response to exertion. Exercise tests are used extensively but they differ so widely in detail that it is difficult to compare results from different laboratories. In general, basic standardization data are not available on energy costs, skill factors, efficiency, and inter- and intra-individual variability.

There is common agreement on the superiority, at least for precise research, of special devices to ensure exact reproducibility of work loads. Of these devices the bicycle ergometer and the motordriven treadmill are the outstanding instruments of choice. Except for cost and portability, the treadmill is preferred to the bicycle ergometer. The main differences are:

1. The work load on the treadmill is fixed without any requirement for the subject to keep time.
2. Skill and apparatus-training factors are at a minimum on the treadmill—everyone knows how to walk.
3. A larger total energy expenditure is obtainable on the treadmill.
4. On the treadmill the work load is automatically adjusted to the body size.
5. Walking is generally a social and occupational necessity so that treadmill measurements provide a basis for evaluation of the handicap of many diseases and injuries.

The present report summarizes the results of a systematic study of treadmill walking at 16 different combinations of speeds and angles covering the range from 2.5 to 4.0 miles per hour at zero to 10 per cent grade.

METHODS. The treadmill used here provides a walking space of 9 feet, 4 inches by 4 feet on a rubber belt sliding on a rigidly supported maple slipway. The belt itself has transverse ridges $\frac{1}{4}$ inch high and $\frac{1}{4}$ inch apart which afford excellent footing. The belt is driven by a 10 horse-power synchronous motor operating through a variable cone transmission (Reeves Co.). The belt pulley tachometer was calibrated for all speeds by linear measurement of belt travel. All grades were checked by direct geometrical measurement and were calculated in terms of vertical climb as per cent of the belt travel. At all times the environ-

¹ This work was done, in part, under a contract, recommended by the Committee on Medical Research, between the University of Minnesota and the Office of Scientific Research and Development. Substantial support was also provided by the National Foundation for Infantile Paralysis, by the Sugar Research Foundation, New York, and by the American Dairy Council, operating on behalf of the National Dairy Association, Chicago.

mental conditions provided a dry bulb temperature of $78^{\circ} \pm 2^{\circ}\text{F.}$, humidity 50 ± 5 per cent of relative saturation, while lighting, noise and air movement were constant. Standard clothing consisted of shoes, socks and cotton shorts.

Oxygen uptake and carbon dioxide output were measured by analysis of the expired air collected through low-resistance connections, in compensated gasometers. The respiration valves used were of the low-resistance diaphragm type and they, and the teeth, enclosed a dead space of only 130 ml. Valves, mouthpiece and connecting tubing were supported by a headgear comfortably fitted to each subject. Gas samples were taken and stored over mercury. All gas analyses were made in duplicate on calibrated Haldane machines checked by daily analyses on outdoor air.

In the principal experiments the respiratory gas exchange was determined in seated rest just before work, twice during a 30 minute period of work and for the first 10 minutes of recovery following work. During work, expired air was collected at 15 to 20 minutes and at 25 to 30 minutes from the start of walking.

All statistical computations on variability were made by the method of the analysis of variance as developed by R. A. Fisher (1936). Calculations of caloric expenditure were made from the Tables of Zuntz as provided by Dubois (1936). No correction was made for nitrogen metabolism. For computation of the oxygen consumption, CO_2 excretion and respiratory efficiency the procedure of Simonson and Hebestreit (1933) was used.

Subjects. The subjects were 54 young men who were found to be free, after careful examination, from signs or significant history of physical or metabolic peculiarity. All were volunteers assigned to this laboratory for Civilian Public Service by the Selective Service System. These men were resident in the Laboratory and were maintained on a controlled "normal" regimen of activity and diet. All of these men were studied at a treadmill speed of 3.5 miles per hour and 10 per cent grade and the effects of training were specifically studied in 8 of the men. Two of the men, A. B. and D. M., were studied on two or more occasions at each of 16 combinations of speed and grade. The subjects A. B. and D. M. had the following characteristics, respectively: age—21 and 25 years; weight—77.0 and 79.5 kgm.; height—172.7 and 184.2 cm.

RESULTS. The most important aspects of the energy expenditure in treadmill walking as of any other exercise are: magnitude of training effects, the effect of the main dependent variables, speed and grade, and reliability and reproducibility of results, computed on the basis of intra- and inter-individual variability. The results will be presented in this order. In addition, some questions of secondary importance for the practical applicability of treadmill walking, such as oxygen debt, will be discussed because of their theoretical interest.

Training effect. The question of the magnitude of training (skill) effects is important at the outset. It is known that it may take weeks, in many types of work, until a uniform level of energy expenditure, i.e., efficiency, is reached. Training effects are of so great a magnitude in bicycle ergometer work that they easily overshadow the effect of all other variables. For practical applicability, then, it is important to know how often the test must be repeated in a given individual in order to achieve maximum skill in performing the task.

The early effects of training were studied on three men. These men were given one brief trial to familiarize themselves with the treadmill and mask; actual collections of expired air were made on the next day. The men walked for one hour each day. Collections were made during the last five minutes of the prescribed walk which was done before breakfast. The oxygen consumption was determined under these conditions at intervals during 25 days of walking. The results are presented in table 1. It is apparent that oxygen consumption measured the second time a man is on the treadmill gives a valid figure which will not be influenced by an increase in the experience of the subject in walking on the treadmill.

It is interesting to note that Knehr, Dill and Neufeld (1942), using a treadmill in which the walking surface was a smooth leather belt supported on a floor of small rollers, observed a significant decrease in oxygen consumption with practice. Although other factors might account for the observed differences during

TABLE 1

The oxygen consumption in cubic centimeters per minute during successive days of treadmill walking at 10 per cent and 2.5 miles per hour of three men unfamiliar with the apparatus. "Days of walking" refers to number of days; each man actually walked for one hour on the treadmill

SUBJECT	DAYS OF WALKING					
	1	3	5	7	10	25
R. S.	1660	1622	1664	1626	1600	1673
E. N.	2001	1996	2022	2035	2016	1950
C. B.	2029	2060	2146	2160	2064	2050
Average	1897	1893	1944	1940	1893	1891

walking at a comparable grade and speed over a six-month period, it is probable that walking on such a surface requires more skill than walking on the treadmill used here.

During a period of 8 months, a group of 7 men walked daily for 1.5 hours on the treadmill at a speed of 3.5 m.p.h. and at 10 per cent grade. Four determinations of the energy expenditure were made (Apr. 22, May 15 and 21, and Dec. 17). The mean values for these 4 determinations were, respectively, 29.13, 27.49, 28.63 and 27.49 cc. of oxygen per minute per kilogram; the least significant difference between any two of these means is, at the 5 per cent level of significance, 0.81 cc. per kilogram per minute, and at the 1 per cent level, 1.11 cc. per minute per kilogram.

A second group of 12 men were similarly "trained" for a period of 3 months. Four determinations (on Nov. 29, Dec. 11, Jan. 2 and Feb. 5) were made; the mean values were, respectively, 27.12, 26.59, 26.72 and 26.29 cc. per minute per kilogram. The least significant differences were 0.46 at the 5 per cent level and 0.62 at the 1 per cent level.

Finally, 2 determinations separated by an interval of 4 to 6 days were made on

36 other men. Here the means were 26.00 and 25.44 and the least significant differences, 0.27 at the 5 per cent level, 0.36 at the 1 per cent level.

There is a statistically significant increase of efficiency, although of small magnitude, from the first to the second trial in all three groups. In the last group of 36 men, the increase of efficiency is about the same as in the second group, although the subjects of the second group walked every day between the trials, while the subjects of the third group did not walk on the days between the two trials. Probably a large proportion of the trend in this case must be ascribed to "technical training," that is, the psychological adaptation of the subject to the mask and other novel features of the experimental situation.

For the further determinations during the training period, the second group shows no significant changes of efficiency, while in the first group there is a significant deterioration of the efficiency on the third trial (May 21) and an improve-

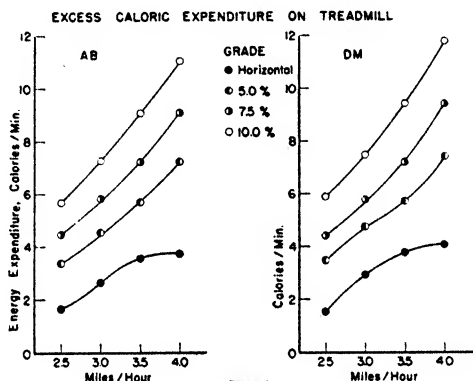


Figure 1.

ment on December 17. Obviously, fluctuations of efficiency may occur in the state of training, due to some unknown factors. The fluctuations, however, are small in magnitude, though they may be similar to the effects of "technical training." In any case, prolonged training does not produce a consistent improvement of efficiency for the duration of several months.

Table 3 (to be discussed later) indicates that the "trial standard deviation," which includes whatever training effect there may be, amounts to less than 3 per cent while the residual or random variability is greater than 2 per cent.

Effect of speed and load. The effect of 16 combinations of speed and grade on the energy expenditure was systematically studied in the two subjects A. B. and D. M.

Figure 1 shows the increase of energy expenditure with speed at the four different grades used. The trend and slope of the curves is similar for both subjects. The slope of the curve for energy output with increasing speed during horizontal walking is rather flat, especially toward the speed of 4.0 m.p.h., for both men. The increase of energy expenditure is much steeper in grade walking, the slope increasing as the grade increases. Linear relationships of the form $O_2 = aS +$

$bG + c$ were fitted to the data by the method of least squares, where O_2 = oxygen consumption in liters per minute; S = speed in miles per hour; G = grade in per cent, and a , b and c are numerical constants. The results provided evidence that the true relationship is curvilinear rather than linear.

Net efficiency. The net climbing efficiency was calculated according to Zuntz. This method has been used by Benedict and Murschhauser (1915), Smith (1922) and others and may be regarded as a generally accepted procedure. The net efficiency of climbing is calculated from the caloric equivalent of the absolute amount of body lift divided by the difference of energy expenditure between grade and horizontal walking. The climbing efficiencies calculated for the subjects in this study are similar to those reported by Smith (1922).

It will be noted in table 2 that the highest efficiency was reached at a grade of 5 per cent for both subjects. A. B. attained a net efficiency of 35.2 per cent at 3.0 m.p.h. while D. M. showed the maximum net efficiency of 40.3 per cent at the higher speed of 3.5 m.p.h. while the efficiency decreases at the lowest and highest speeds. The greatest spread of efficiencies is seen at the medium speeds.

TABLE 2
Net efficiencies of grade walking

SUBJECT	2.5 M.P.H.			3.0 M.P.H.			3.5 M.P.H.			4.0 M.P.H.		
	5.0%	7.5%	10%	5.0%	7.5%	10%	5.0%	7.5%	10%	5.0%	7.5%	10%
A. B.	31.7	31.4	30.0	35.2*	34.0	31.9	35.0	34.2	31.2	24.8	26.5	26.5
D. M.	28.3	31.6	28.7	37.6	38.7	33.2	40.3*	37.3	31.2	26.6	27.3	25.9

Values expressed as per cent efficiency.

* Highest level of efficiency.

It is of interest that D. M. shows in general higher climbing efficiencies than A. B. in spite of the fact that his absolute energy expenditure is higher in most variations (fig. 1). This apparent higher net climbing efficiency is due to the fact that his rate of energy expenditure during horizontal walking is relatively higher than during grade walking compared to A. B. It would appear that objections could be made against expressing net efficiency by such a method of calculation. The separation of grade walking into horizontal and vertical components for the purpose of expressing mechanical efficiency is obviously not compatible with the mechanics of motor co-ordination. It is to be expected that if a separation of the horizontal component and vertical component were possible, the climbing efficiency would depend on the vertical speed rather than on the horizontal speed. Actually the climbing efficiency depends on the horizontal rather than on the vertical speed. It is believed that grade walking is an integrative complex phenomenon where a component of the forward movement is used for climbing and vice versa. Further evidence for this view will be given in a series of studies on poliomyelitis patients which will be reported elsewhere.

Variability. The reproducibility and, consequently, the reliability of results is dependent on the interference of uncontrolled or uncontrollable factors. The

analysis below isolates the several sources of variability which can be identified in a study of this type. The factors which have to be considered are: 1, variability within the same experiment; 2, daily variations, and 3, inter-individual variability. Intra-individual variability of types 1 and 2 will be studied mainly in the two subjects A. B. and D. M., for all combinations of speed and grade, and also in a larger group of 47 subjects. In this latter group, we may also study inter-individual variability.

Variability within an experiment. The standard deviation between 64 pairs of oxygen consumption determinations taken at 15–20 minutes and 25–30 minutes after the beginning of walking was ± 44.6 cc. of oxygen, or 3.12 per cent of the mean value for 128 determinations. It differed only slightly between A. B. and D. M. (2.94 per cent and 3.29 per cent respectively). This value is only slightly greater than the technical error of measurement, which amounts to about 1 per cent; thus it is reasonable to assume that a steady state was maintained in each of the arrangements.

Variability between days. The standard deviation between repetitions of different days amounted to 2.57 per cent of the mean. Using the methods of analysis of variance, the conclusion was drawn that this value was not significantly different from the value of 3.12 per cent for variability within an experiment. Hence, these two kinds of variability may be attributed to a common source, whose standard deviation was found to be 2.95 per cent of the grand mean. Further analysis showed that this value did not change with speed or grade. It may be taken as the absolute error of a single measurement.

Variability between men. If the graphs in figure 1 for the two men, A. B. and D. M., were superimposed in such a way as to bring the means for the two men into coincidence (which amounts to displacing A. B.'s graph upward 0.26 cal.) it would be found that corresponding points would be very close together. This brings out very clearly the similarity of the trend patterns for the two men. Numerically, we find that the variability between the two individuals, after allowance has been made for the difference of 0.26 cal. in mean oxygen consumption, is only 3.27 per cent.

Dependent variability due to change of speed and grade. For the question of reliability of results the relationship between the changes produced by the controlled independent variables and the random variations produced by uncontrolled factors is of fundamental importance. The random variability within the same experiment (about 3 per cent) or between days (2.6 per cent) should be small compared to the changes produced by change of the dependent variables, speed and grade. When comparing the extremes of 2.5 m.p.h. and zero grade with 4 m.p.h. and 10 per cent grade, the average total energy expenditure increased by 460 per cent and the excess expenditure due to walking by 615 per cent. It is evident that the variation due to the controlled factors of speed and grade is very large when compared with the variation due to uncontrolled factors.

Inter-individual and intra-individual variability. Table 3 presents the values for the inter-individual variability of a group of 47 subjects walking at a speed of 3.5 m.p.h. and 10 per cent grade. Since each subject was investigated two to

four times, it was possible to break down the gross variability into three components each computed as a standard deviation (S.D.): 1, inter-individual S.D., i.e., standard deviation between individuals for an average trial; 2, trial S.D., i.e., between the experiments performed on different days, for an average individual; 3, residual (error) S.D. (Fisher, 1936; Snedecor, 1940). The calculations were performed both for the gross oxygen consumption and for the oxygen consumption per kilogram body weight. The particular combination of speed and grade used (3.5 m.p.h., 10 per cent grade) is at the upper end of the range used in this Laboratory. The effect of expressing oxygen consumption related to body weight is very apparent in table 3. In the case of gross oxygen consumption, the individual S.D. is much greater than the trial S.D. or the error S.D. The introduction of the body weight has the effect of decreasing the individual S.D. very markedly, while trial S.D. and error S.D. remain essentially the same. It may be noted that the oxygen consumption values for the subjects A. B. and D. M., at 3.5 m.p.h. and 10 per cent grade, fall in the middle of the range for the 47 subjects.

TABLE 3

Inter- and intra-individual variability of the oxygen consumption of 47 subjects during walking on the treadmill at a speed of 3.5 m.p.h. and 10 per cent grade

	GROSS	OXYGEN CONSUMPTION PER KILOGRAM BODY WEIGHT
Mean ml./min.....	1839.2	25.97
Per cent of the mean:		
Individual S.D.....	9.37	3.99
Trial S.D.....	2.68	2.50
Error S.D.....	2.17	2.11

Individual S.D. = Standard deviation between individuals for an average trial.

Trial S.D. = Standard deviation between trials for an average individual.

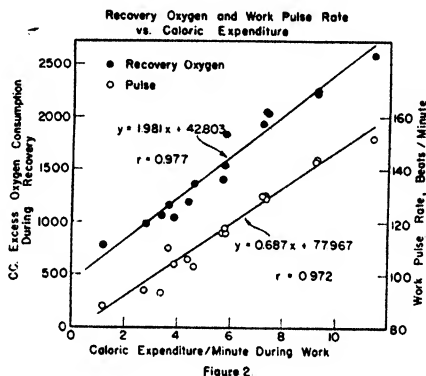
Error S.D. = Residual error in a table of trials \times individuals. It is that component of variation in such a table which is not attributable either to trial or to individual variations.

A group of 7 men was investigated at the lowest speed of 2.5 m.p.h. with the treadmill horizontal. The energy expenditure was converted to calories per horizontal kilogrammeter. The standard deviation for this group was found to be 15.1 per cent of the mean. When this value is compared to the standard deviation found for oxygen consumption referred to the body weight in table 3, it is noted that the variability is much greater for the slower speed. It may be concluded that energy metabolism of walking is a more stable function at a higher speed and grade.

The mean value for these seven men is 0.434 Cal. per horizontal kilogrammeter at a speed of 2.5 m.p.h. (67 meters per min.). Subject D. M. is very near the mean (0.464 Cal. per horizontal kilogrammeter) and A. B. below the mean (0.320 Cal. per horizontal kilogrammeter). These values are fairly comparable to similar values for horizontal walking found in a review of the literature by Benedict and Murschhauser (1915).

Excess oxygen consumption of recovery. The average 10 minute excess oxygen consumption of recovery (oxygen debt) was plotted against average excess caloric expenditure per minute of work, independent of grade and speed. This is shown in figure 2. It is to be noted that there is a linear increase of excess oxygen consumption of recovery with increasing energy expenditure. The product moment correlation coefficient for these two functions is 0.977 with a t-value of 16.90, indicating a highly significant relationship.

That the greater part of recovery is completed in the first three minutes after work was shown in two exploratory experiments. In subjects A. B. and D. M. the 10-minute recovery was divided into two parts of three minutes and seven minutes. In the case of A. B., 96 per cent of the excess oxygen consumption of recovery was used in the first three minutes. Subject D. M. completed 86 per cent of the excess oxygen consumption for the 10-minute period in the first 3 minutes. Since the major part of the recovery is completed within the first few



minutes after work, it is safe to assume that a standardized period of 10 minutes of recovery oxygen consumption adequately measures this function within the range of our experimental arrangements.

Energy expenditure as related to heart rate. Figure 2 shows the average number of heart beats per minute during work plotted against the average excess energy expenditure during work independent of grade and speed. The product moment correlation coefficient is 0.972 with a t-value of 15.35, which is highly significant. This value is in conformity with the findings of C. Taylor (1941) for a different kind of work. This investigator found correlations of 0.969 and 0.955 between heart rate and work load with two subjects working on a bicycle ergometer. It is understood that the close correlation between pulse rate and oxygen consumption was obtained in a constant state of training, as shown by the following observations.

Pulse rates were counted on 8 men during the first 14 times they walked on the treadmill. Consistent decrease in pulse rate for all 8 men does not appear to begin until trial 5, that is, the first four trials were without consistent trend while

on the fifth trial all 8 men had a significantly lower pulse than on the fourth. Between trials 4 and 8 the greatest decrease in pulse rate took place and these trials show a highly significant trend. The last 7 trials were completely without trend. We may conclude that subjects A. B. and D. M. had reached a plateau for pulse rate after the 10-day preliminary training period.

Number of steps per minute. Table 4 shows the average number of steps per minute and the stride (cm.) for the four steps and grades. It can be seen that the rate of steps and the stride increase with the speed almost independent of the grade. Obviously, grade is not an important factor for stride with the limits of horizontal walking and 10 per cent grade. The increase of the rate of steps with increasing speed is more pronounced than the increase of the stride. The rate

TABLE 4
Average rate (per minute) and stride (cm.) of steps

SUBJECT	GRADE	SPEED							
		2.5 m.p.h.		3 m.p.h.		3.5 m.p.h.		4.0 m.p.h.	
		Rate	Stride	Rate	Stride	Rate	Stride	Rate	Stride
A. B.	%								
	0	100	67	112	72	122	77	130	83
	5	95	71	112	72	121	78	131	82
	7.5	92	73	109	74	122	77	128	84
	10	99	68	112	72	124	76	131	82
Average.....		96	70	111	73	122	77	130	83
D. M.	0	92	73	100	80	108	87	115	93
	5	88	76	89	81	108	87	116	92
	7.5	87	77	96	84	108	87	116	92
	10	90	74	99	81	109	86	119	90
Average.....		89	75	99	81	108	87	116	92

of A. B. is consistently higher and consequently the stride smaller at each speed. This fact is probably accounted for in that D. M. has longer legs than A. B.

Respiratory efficiency. The ability of the body to remove oxygen from the air breathed is expressed as the per cent of oxygen in the expired air corrected for equality of the inspiratory and expiratory volumes. These figures are presented in table 5. An increase in respiratory efficiency corresponds to the increase in energy output for A. B. A trend is noted which indicates deterioration of respiratory efficiency with higher metabolic rates for D. M. as was seen for A. B. This deterioration of respiratory efficiency with higher work loads has been demonstrated by C. Taylor (1941) in the work experiments on the bicycle ergometer.

DISCUSSION. Relating the oxygen consumption of work to the body weight is an accepted procedure to express walking efficiency. It was shown in the data

for 47 subjects that this procedure removes one variable factor and decreases the percentage variability in a group of men with a wide range of body weight.

The data presented above suggest that further studies are necessary for better understanding and interpretation of the mechanical efficiency of climbing. Detailed motion analysis seems to be a feasible approach to this problem.

This study is a standardization background for treadmill walking and has application for the study of fitness. Several advantages of this type of work have been demonstrated. There is little training effect. The small intrinsic variations imply good reproducibility. Comparatively small variations of speed and grade produce accurately measurable differences of energy expenditure, hence this type of work is useful in establishing trends of caloric expenditure. There is a prolonged steady state maintained with minor variations. The data presented include a rather large experimental group, so that comparison of the normal levels and trends of energy metabolism to disabled patients such as poliomyelitis patients can be made on a reliable basis. It has been shown that the treadmill has the possibility of wide application in fitness testing.

TABLE 5
Respiratory efficiencies

SUBJECT	2.5 M.P.H.				3.0 M.P.H.				3.5 M.P.H.				4.0 M.P.H.			
	Horiz.	5.0%	7.5%	10%	Horiz.	5.0%	7.5%	10%	Horiz.	5.0%	7.5%	10%	Horiz.	5.0%	7.5%	10%
A. B.	4.72	4.82	4.99	5.30	4.78	4.98	5.14	5.21	4.88	5.10	5.21	5.40	4.86	5.14	5.12	5.22
D. M.	5.03	4.86	4.80	5.02	4.95	5.01	5.00	5.12	4.93	4.80	4.98	4.99	5.03	4.98	4.89	4.67

Respiratory efficiency = per cent of O₂ of inhaled air minus per cent of O₂ in expired air corrected for inspiratory and expiratory volumes. All values in the table are percentages.

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SUMMARY

1. The energy cost of treadmill walking was studied with 2 normal young men at all combinations of speeds 2.5, 3.0, 3.5 and 4.0 miles per hour and grades 0, 5.0, 7.5 and 10 per cent. In 128 measurements the replicate variability of the measurement was 2.95 per cent of the grand mean and independent of speed and grade. It may be compared with the effect of changing from 2.5 m.p.h. and zero grade to 4.0 m.p.h. and 10 per cent; this change involved a mean increase of energy expenditure of 460 per cent.

2. The curves for increase in energy expenditure with speed at the 4 different grades were substantially identical in these 2 subjects.

3. The energy cost of treadmill walking at 3.5 m.p.h. and 10 per cent grade was measured in 47 additional subjects. The inter-individual standard deviation was 9.37 per cent of the gross oxygen consumption; this was reduced to 3.99 per cent when calculated per kilogram of body weight.

4. Practice and training on the treadmill was studied in from 2 to 240 daily trials in 3 different groups comprising a total of 55 men. There were traces of a trifling increase in walking efficiency with training but in all cases this was less than the replicate variability.

5. The inter-individual variability of low rates of work were compared to the variability for high rates of energy expenditure. The higher rates were more stable from individual to individual.

6. Net climbing efficiencies showed maxima of 35 to 40 per cent at medium speeds. This kind of calculation is discussed critically.

7. The relation between excess oxygen consumption in recovery and the excess energy expenditure during work was studied. Without regard to speed and grade a product-moment coefficient of correlation of 0.977 was found.

8. The relation between the average pulse rate in work and the excess energy expenditure during work was studied. Without regard to speed and grade a product-moment coefficient of correlation of 0.972 was found.

9. The stride length was found to be dependent on speed but practically independent of grade.

10. The data are presented to form a standardized background for the application of the treadmill as a device for studies on controlled work output.

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THE EFFECT OF LOW AND HIGH CARBOHYDRATE MEALS ON THE BLOOD SUGAR LEVEL AND ON WORK PERFORMANCE IN STRENUOUS EXERCISE OF SHORT DURATION^{1, 2}

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Functional hypoglycemia, which in its milder forms gives rise to such symptoms as a sense of weakness and fatigue, appears to be a well established clinical entity. There are some who believe that hypoglycemia with its accompanying symptoms may be a fairly common occurrence amongst the population at large (1, 2). In the treatment of functional hypoglycemia good results have been obtained by restricting the carbohydrate intake and placing the patient on a diet containing a high percentage of protein and fat (1, 3, 4). This has suggested to some investigators that a diet containing a relatively large amount of sugar or other readily absorbable carbohydrate, supplies an additional insulogenic stimulus which brings about hyperinsulinism and a resultant hypoglycemia. On the basis of this reasoning the assumption has been made (1, 2) that a typical American breakfast and luncheon which contain a large amount of carbohydrate are conducive to the lowering of the blood sugar level several hours later with concomitant hypoglycemic symptoms of hunger and weakness and an impairment of one's capacity for work.

A search of the literature failed to reveal any substantial evidence for this belief. In a study on one subject remaining in bed throughout the experiment, Thorne, Quinby and Clinton (2) found that the blood sugar fell from a pre-ingestion level of approximately 85 mgm. per cent to 69 mgm. per cent two hours after a meal containing a large percentage of carbohydrate. Simultaneously with the drop in blood sugar the subject complained of hunger and weakness. When a high fat meal of the same caloric value was ingested, the blood sugar fell more slowly. At the end of five hours it was 71 mgm. per cent and although the subject experienced hypoglycemic symptoms at this time they were not as pronounced as when the carbohydrate meal had been taken. After a high protein meal the blood sugar remained at a normal level with the subject reporting a feeling of well-being throughout the six hour experimental period. Obviously no generalizations can be drawn from these observations on a single subject. The experimental conditions were definitely not representative of normal living conditions; furthermore, one might hesitate to designate a blood sugar level of 69 and 71 mgm. per cent as a clear cut hypoglycemia.

Other investigations have been reported on the blood sugar level following the ingestion of sugar and other carbohydrates (5, 6), but these were not concerned

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² Acknowledgment is made to the Sugar Research Foundation for a grant-in-aid for conducting this investigation.

with the question of hunger, weakness and fatigue nor with the effect of isocaloric meals of different composition on the blood sugar concentration.

The present study was undertaken with two objectives in mind: *a*, to obtain comparative data on the blood sugar level of a large number of normal individuals engaged in their usual activities after eating a low and a high carbohydrate meal; *b*, to determine whether a high carbohydrate meal is conducive to the hypoglycemic syndrome several hours later or to an impairment in one's capacity for strenuous work.

PART 1. Procedure. Fifty-nine medical students served as volunteer subjects. Among the group there were the variations in dietary habits that might be expected when a number of individuals are selected at random. Some were accustomed to eating a breakfast containing a large amount of protein and fat, others preferred more carbohydrate, while a few ordinarily ate no breakfast at all.

For four consecutive mornings the subjects ate a breakfast containing a high percentage of fat and protein, and the next week a high carbohydrate breakfast for four days, the composition of which is presented in table 1. The meals were served from 7:30 to 8:00 a.m. and approximately three hours later the subjects reported at the laboratory for a finger puncture, having pursued in the meantime their usual activities. The blood was analyzed for sugar by the Hagedorn-Jensen procedure (7). At the time of the withdrawal of the blood samples each subject recorded in writing a brief statement of how he felt.

Results. The average sugar content of the blood after the low carbohydrate meal was 101 mgm. per cent and after the high carbohydrate meal 99 mgm. per cent. These averages were obtained from 215 and 210 determinations, respectively. There were scheduled 236 analyses after each type of meal, but occasionally a subject failed to report to the laboratory. In no instance was the blood sugar less than 80 mgm. per cent.

In most instances the subjects stated that they felt the same as usual after both kinds of breakfast. After the low carbohydrate meal, 9 per cent of the reports carried the notation "hungry" or "slightly hungry." On the days when the high carbohydrate breakfast was taken, 28 per cent of the statements recorded "hunger" and 6 per cent more "hunger and weakness." There was no correlation, however, between the subjective state and the blood sugar level. Obviously, the sense of hunger and weakness could not have been related to low blood sugar for, as stated previously, there were no values below 80 mgm. per cent. Hunger and weakness were frequently experienced only on the first day after the high carbohydrate breakfast and in some instances on only the 2nd, 3rd or 4th day.

PART 2. Procedure. In this group of experiments the procedure was exactly the same as in the preceding ones except that the breakfasts (see table 2) contained 600 instead of 400 calories and that three blood samples were drawn instead of one. The blood samples were obtained one, two, and three hours after breakfast. Fifty medical students, twenty of whom had taken part in the preceding experiments, served as subjects. Two experiments were conducted on each subject with each type of meal. A report of how the subject felt was recorded at the time of withdrawal of the blood samples.

Results. The blood sugar level one, two, and three hours after the low carbohydrate meal was 116, 109, and 105 mgm. per cent, respectively; after the high carbohydrate breakfast it was 132, 113, and 100 mgm. per cent, respectively. Each value is an average of 100 determinations. While the blood sugar concentration was appreciably higher one hour after the high carbohydrate meal than at the same interval following the low carbohydrate breakfast, it should be noted that it was at a normal level two hours later. The blood sugar level at the end of the third hour was practically the same as in the preceding experiments in which a much smaller breakfast was ingested.

Of the 300 individual reports of how the subjects felt at the time that the blood samples were withdrawn, there were only 9 in which the subject stated that he

TABLE 1
Composition of breakfasts yielding approximately 400 calories

FOOD	SERVING	CHO	PROTEIN	FAT	CALORIES
High carbohydrate breakfast					
Corn flakes	30 grams	26	2	0	112
Toast	1 slice	13	2	0	60
Sugar	28 grams	28	0	0	112
Skim milk	$\frac{1}{2}$ pint	11	9	2	98
Total		78	13	2	382
Total calories		312	52	18	382
% of total caloric intake		81	14	5	100
Low carbohydrate breakfast					
Eggs (boiled)	2	0	13	10	142
Toast	2 slices	26	5	0	124
Bacon	2 strips	0	4	5	61
Butter	7 grams	0	0	6	54
Total		26	22	21	381
Total calories		104	88	189	381
% of total caloric intake		27	23	50	100

felt "hungry" or "slightly hungry" after eating the high carbohydrate meal. The blood sugar at the time when the subjects said they were hungry ranged from 90 to 120 mgm. per cent. Following the low carbohydrate meal there were no complaints of hunger.

The fewer instances in which hunger was experienced in this group of experiments can probably be accounted for by the larger food intake than in the preceding experiments. It is questionable, however, whether the hunger was due to physiological conditions, such as the emptying of the stomach, or to psychological factors. It is of interest to note that one subject said that he was hungry at nine, ten, and eleven o'clock after a high carbohydrate breakfast, but was not hungry one week later following a breakfast identically the same.

These data show that in normal individuals an average size breakfast of high carbohydrate content is not conducive to the hypoglycemic syndrome several hours later.

PART 3. Procedure. In the preceding experiments sugar determinations were made only on blood from the finger tip which has the same sugar concentration as arterial blood (8). This procedure was followed because it is our opinion that in a study of this kind interest should be centered primarily on the amount of sugar in the blood that is available to the tissues. However, since exception may be taken to this point of view, a series of experiments was run on thirty-eight additional subjects in which venous and finger tip blood samples were drawn

TABLE 2
Composition of breakfasts yielding approximately 600 calories

FOOD	SERVING	CHO	PROTEIN	FAT	CALORIES
High carbohydrate breakfast					
		<i>grams</i>	<i>grams</i>	<i>grams</i>	
Corn flakes.....	30 grams	26	2	0	112
Orange juice.....	200 cc.	20	2	0	88
Toast.....	2 slices	26	5	1	133
Skim milk.....	$\frac{1}{2}$ pint	11	9	2	98
Sugar.....	28 grams	28	0	0	112
Jelly.....	1 T.	16	0	0	64
Total.....		127	18	3	607
Total calories.....		508	72	27	607
% of total caloric intake.....		84	12	4	100
Low carbohydrate breakfast					
Bacon.....	4 strips	0	7	10	118
Butter.....	21 grams	0	0	17	153
Eggs.....	2 boiled	0	13	10	142
Toast.....	3 slices	38	7	1	189
Total.....		38	27	38	602
Total calories.....		152	108	342	602
% of total caloric intake.....		25	18	57	100

practically simultaneously, one, two, and three hours after the low and the high carbohydrate meals in table 2. Half the number of venous samples were drawn immediately before and half immediately after the finger puncture. Venous blood was analyzed for sugar by the Shaffer-Hartmann-Somogyi procedure and finger tip blood by the Hagedorn-Jensen method (7).

Results. The results in this series of experiments were substantially the same as in the preceding ones. The average blood sugar concentration in the finger tip blood was 139, 120, 108 mgm. per cent and 117, 114, 108 mgm. per cent after the high and the low carbohydrate meals, respectively. The venous blood sugar was 117, 114, 108 and 85, 83, 85 mgm. per cent, respectively. These averages are within what is usually regarded as the normal range. If a comparison is to be

instituted between the arterial and venous sugar concentrations, a correction factor should be applied to these values, as in our laboratories the Hagedorn-Jensen method yields results on the average 15 mgm. per cent higher than the Shaffer-Hartmann-Somogyi procedure on the same blood sample.

The concentration of sugar in ninety-two per cent of the venous samples was above 70 mgm. per cent. In the remainder it ranged from 60 to 70 mgm. per cent with no regularity as to time after the meal. Other investigators (9) also have found that the sugar concentration may be as low as 60 mgm. per cent in the venous blood of normal, healthy individuals. These observations would indicate that a venous blood sugar value in the neighborhood of 60 mgm. per cent cannot be taken as *prima facie* evidence of physiological abnormality. The low values in our experiments occurred in about the same frequency following the low and the high carbohydrate meals. There was no relationship between the sense of hunger and the blood sugar level.

PART 4. There remained to be considered the possibility that hypoglycemia might occur after a larger meal containing more carbohydrate than was ingested in the previous experiments, and that one's capacity for work might be affected by the products of digestion at the organism's disposal after various kinds of meals, regardless of the blood sugar level.

In studying the work capacity of the human organism the experimenter is confronted with the difficulty that the subject may not exert himself to the utmost and that he may diminish or discontinue his efforts before he is actually exhausted. This difficulty can be surmounted by selecting a form of exercise, such as hard, fast swimming for a hundred yards. This kind of exercise is extremely strenuous. Swimming at a speed faster than two feet a second raises the metabolism to more than ten times the basal level and at speeds beyond five feet per second, more than 100 times the basal. Unskilled swimmers expend from 2 to 5 times as much energy as skilled swimmers (10). The exertion is also exhausting. Most swimmers, particularly those who are not expert, feel during the last lap of a hundred yard sprint that they will become completely exhausted before they reach the goal. Yet, it is not a difficult matter to induce one who is interested in swimming and in improving his performance to put forth a maximum effort for the short period of time that it takes to swim this distance. The length of time required to swim the first lap under various experimental conditions offers a means of comparative observations on the subject's general physical condition at the outset of exercise, while the drop-off or deceleration of speed in the second and third laps serves as an objective index of the fatigue that occurs in the course of the exercise.

Procedure. Twelve subjects in the Navy V-12 program at Emory University served as subjects of the experiment. They were all deeply interested in competitive swimming and anxious to improve their performance. None, however, were expert swimmers. Ten swam free style, one the breast stroke and one the back stroke. The same subjects were used in all the experiments, each one serving as his own control. One found it necessary to drop out at the end of the fourth experiment, leaving only eleven for the last four experiments. Although

all the subjects were in splendid physical condition and in training, they were nevertheless subjected to a week of further intensive training before beginning the experiment. Their other activities were fairly uniform from day to day except for Saturdays and Sundays, but no experiment was conducted on these days.

TABLE 3

Composition of high carbohydrate meal eaten by subjects 2½ to 3 hours before swimming

FOOD	SERVING	CHO	P	F	CALORIES
		grams	grams	grams	
Grape fruit juice.....	1 glass (227 grams)	22	1	0	92
Corn flakes.....	30 grams	26	2	0	112
Sugar.....	35 grams	35	0	0	140
Potato (baked).....	150 grams	28	3	0	124
Bread.....	2 slices	26	5	1	133
Steak.....	2 oz.	0	16	5	109
Jelly.....	1½ T. (31 grams)	25	0	0	100
Butter.....	7 grams	0	0	6	54
Lettuce.....	2 leaves (shredded)	2	1	0	12
Banana.....	1	28	2	1	129
Skim milk (for cereal).....	150 cc.	8	5	0	52
Total.....		200	35	13	1057
Total calories.....		800	140	117	1057
% of total caloric intake.....		76	13	11	100

TABLE 4

Composition of low carbohydrate meal eaten by subjects 2½ to 3 hours before swimming

FOOD	SERVING	CHO	P	F	CALORIES
		grams	grams	grams	
Steak.....	10 oz.	0	82	26	562
Bread.....	1 slice	13	2	0	60
Egg (hard boiled).....	1	0	7	5	73
Butter.....	14 grams	0	0	12	108
Green peas.....	4 oz.	11	4	0	60
Tomato.....	1 slice	1	0	0	4
Mayonnaise (on tomato).....	2 soup spoons (level)	0	1	16	148
Peaches (canned).....	1 half	8	1	0	36
Total.....		33	97	59	1051
Total calories.....		132	388	531	1051
% of total caloric intake.....		13	37	50	100

A specially prepared meal was served at a separate table in the mess hall on the days of the experiments. A high and a low carbohydrate meal was served alternately for successive experiments. The constituents of the meals prepared for the last four experiments are given in tables 3 and 4. In the first four experiments the meals contained 1300 calories with the items of food and the percentage of carbohydrate, fat, and protein the same as in the tables. The caloric

content was reduced in the later experiments because the subjects complained of a somewhat uncomfortable sense of fullness after the heavier meal. All the subjects reported complete satisfaction with the lighter meal.

The meals were served at 4:45 p.m. and the swimmers reported at the pool at 7:00 p.m. Scheduled class work made it impossible to conduct the experiment at any other time of the day. A blood sample was obtained from a finger puncture and then each swimmer "warmed up" by swimming slowly 100 yards. A brief rest followed and after this the subjects were taken in order, each one swimming and timed separately from the others. At a given signal the swimmer toed the mark and dove off as in a regular swimming meet. The subject was

TABLE 5

*Swimming time and blood sugar levels after high and low carbohydrate meals**

Exp't no.	TIME				DROP-OFF		BLOOD SUGAR	
	1st 33½ yards	2nd 33½ yards	3rd 33½ yards	100 yards	2nd 33½ yards	3rd 33½ yards	Before swim	After swim
High carbohydrate meal								
	seconds	seconds	seconds	seconds	seconds	seconds	mgm. %	mgm. %
1	19.3	24.4	28.8	72.5	5.1	9.5	124	141
3	18.9	24.2	28.6	71.7	5.3	9.7	123	137
5	19.2	24.4	28.0	71.6	5.0	8.8	121	136
7	19.1	24.2	28.7	72.0	5.1	9.6	124	139
Avg.....	19.2	24.3	28.5	72.0	5.1	9.4	123	138
Low carbohydrate meal								
	seconds	seconds	seconds	seconds	seconds	seconds	mgm. %	mgm. %
2	19.1	24.3	29.4	72.8	5.2	10.3	117	143
4	19.2	24.4	28.9	72.5	5.2	9.7	116	134
6	18.9	23.9	28.4	71.2	5.0	9.5	119	136
8	19.0	24.4	28.5	71.9	5.4	9.5	113	131
Avg.....	19.0	24.2	28.8	72.1	5.2	9.8	116	136

* Each figure in groups 1-4 is an average on 12 swimmers and in groups 5-8 on 11 swimmers. Final averages were accordingly derived from 46 individual experiments.

always intent on making a new record for himself. The swimming coach and one of us (J. H.) followed the swimmer alongside the pool, exhorting him to put forth his maximum effort. The three laps of 33½ yards were each timed in split seconds. Three to five minutes after the sprint a second blood sample was taken.

Results. There was no significant difference in the drop-off in the second and third laps as compared with the first on the two types of meals (see table 5). The blood sugar was slightly though definitely higher after the swim in all but 6 out of 82 experiments. It never fell below 100 mgm. per cent except in two instances after the low carbohydrate meal when it was 97 mgm. per cent immediately after swimming.

Swimmers like other athletes vary in their performance from day to day, but inspection of the averages obtained on different days under identical conditions

(table 5) shows that the number of subjects used in these experiments was sufficient to make allowance for normal variations and to provide averages that are representative values for the group. The final averages in the table are derived from all the data obtained in the experiments with both the lighter and the heavier meals. This procedure is justifiable in as much as the difference of 300 calories, as shown by the data on the separate groups of experiments, had no effect on the performance of the swimmers.

From these experiments on the swimmers, it may be concluded that a high carbohydrate meal is not conducive to an impairment in one's capacity for exhausting work of short duration. The fact that the time required to swim the first lap was the same in all experiments indicates that there was no difference in the effects of the two types of meals with regard to the fatigue of the swimmers at the outset of exercise. The drop-off in the second and third laps as compared with the first shows that after the high carbohydrate meal the subjects did not become more fatigued in the course of swimming than after the low carbohydrate meal.

DISCUSSION. These observations indicate that the hypoglycemic syndrome of functional origin is a pathological condition and not a normal physiological response to a high carbohydrate intake. Were it not an abnormal condition, some evidence of the syndrome should have been observed in our large group of normal, healthy subjects. It has been assumed that the mechanism responsible for the low blood sugar in functional hypoglycemia is the same as that which accounts for the drop in blood sugar two to three hours after the oral administration of sugar in the dextrose tolerance test. The secretion of insulin is generally believed to be governed by the level of the blood sugar concentration (11, 12, 13). On the basis of this premise one explanation of the normal glucose tolerance curve attributes the drop in blood sugar to an increased secretion of insulin. Others hold that the hypoglycemic phase of the curve is due to a decrease in the supply of sugar to the blood by the liver in response to an influx of exogenous sugar (14, 15). If a high carbohydrate intake should prove to be the primary etiologic factor in the clinical syndrome of functional hypoglycemia, then it would appear that there should be postulated either a hypersensitivity of the islet tissue in those individuals who manifest this disorder, or a derangement in the blood sugar homeostatic mechanism of the liver.

Grateful acknowledgment is made to the subjects who took part in the experiments, to Commander McCann, Executive Officer of the V-12 unit at Emory University, to Thomas McDonough, Professor of Physical Education, and to Ed Shea, Swimming Coach, without whose co-operation it would have been impossible to conduct this investigation.

SUMMARY AND CONCLUSIONS

Blood sugar determinations were made on finger tip blood of fifty-nine normal, healthy young adults two and a half to three hours after a low and a high carbohydrate breakfast containing approximately 400 calories.

There was no appreciable difference in the blood sugar level after the two types of meals.

Blood samples were obtained by the same procedure on 50 subjects one, two, and three hours after a low and a high carbohydrate breakfast containing 50 per cent more calories than the breakfast ingested in the preceding experiments.

On thirty-eight additional subjects venous and finger tip blood was drawn simultaneously.

The average blood sugar (arterial and venous) was within the normal range. In a few instances the sugar concentration of venous blood fell below 70 mgm. per cent, but these low values, which are regarded as normal deviations, occurred in about equal frequency after the low and the high carbohydrate meals. There was no relationship between the blood sugar level and the sense of hunger and weakness.

The performance of swimmers in a hundred yard sprint was the same two and a half to three hours after a high carbohydrate meal and an isocaloric meal containing a low percentage of carbohydrate.

There was no difference in the blood sugar level two and a half to three hours after these low and high carbohydrate meals. Immediately after swimming, the blood sugar concentration was higher than before swimming but was the same on the two types of meals.

A high carbohydrate meal is not conducive to hypoglycemia and its usual symptoms of hunger, weakness and fatigue several hours later in normal, healthy individuals.

It is concluded that the hypoglycemic syndrome that is relieved by restriction of the carbohydrate intake is a pathological condition and not a normal physiological response to a high carbohydrate meal.

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THE EFFECT OF DIETHYLSTILBESTROL ON THYROIDECTOMIZED RATS¹

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The administration of the synthetic estrogen, diethylstilbestrol, as well as thyroidectomy, produces certain marked morphological and physiological changes in the experimental animal. Among the changes which investigators have found to result from thyroidectomy, are lowering of the basal metabolic rate, slowing of growth, modification in the structure of the pituitary and a decrease in the fertility rate. Diethylstilbestrol, on the other hand, produces marked changes in the reproductive organs, stunts the growth and causes hypertrophy of the adrenals and pituitaries of certain types of experimental animals. It also produces an elevation in the liver glycogen in the rat, and in some cases reduces the requirement of insulin in human diabetics.

Diethylstilbestrol has been administered to thyroidectomized rats in order to determine its effect on certain phases of carbohydrate metabolism as well as its effect on the morphology of certain organs.

METHODS. Female rats of the Long-Evans strain were used in this study. The type and length of treatment was varied, as shown by the following groups:

1. Rats that had been thyroidectomized for 8 months were given daily subcutaneous injections of 100 micrograms of diethylstilbestrol for the last 20 days of the eighth month.

2. Rats that had been thyroidectomized for 8 months were given subcutaneous implants of two 10-mgm. pellets of diethylstilbestrol (95 per cent diethylstilbestrol and 5 per cent methyl cellulose). The first pellet was implanted at the time of thyroidectomy and the second 6 months later.

3. Rats that had been thyroidectomized for 10 days were given daily injections of 100 micrograms of diethylstilbestrol for the following 20 days.

Control rats for both thyroidectomized and normal groups were studied.

At the end of each experiment the animals were fasted for 36 hours. Immediately before anesthetizing with sodium amytal, blood samples were taken from the tail vein for determination of blood sugar by the Shaffer-Hartman-Somogyi micro-method. In certain cases blood N.P.N. values were determined using the method of Koch-McMeekin. Following anesthetization, the gastrocnemii and samples of liver were removed, then frozen quickly in a mixture of carbon dioxide and ether in preparation for the determination of glycogen levels by the method of Good, Kramer and Somogyi. Urine was collected in 5 per cent sulfuric acid during the last 24 hours of the 36 hour fast. Before the animals were

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placed in the metabolism cages at the end of the fast, the urinary bladders were emptied by applying external pressure. Determinations of urinary nitrogen were made by the micro-Kjeldahl method or by Nesslerization. The gonads, adrenals, pituitaries and kidneys were removed, weighed and, together with pieces of liver, were placed in appropriate fixatives for microscopic examination. A check made for thyroid remnants in the operated animals disclosed a small amount of regenerated tissue in three rats. These animals were included in the study, however, since they showed no differences from those in which the operation was complete.

RESULTS. A. *Glycogen and Blood Sugar.* Rats which were given relatively large doses of diethylstilbestrol had a mild anorexia, generally lost weight for 3

TABLE 1
Glycogen, blood sugar and organ weights
(Female rats, Long-Evans strain)

TREATMENT	NUMBER ANIMALS	WT. IN GRAMS AT END OF 36 HR. FAST	BLOOD SUGAR	LIVER GLYCOGEN	MUSCLE GLYCOGEN	PITUITARY MGM. /100 GRAMS BODY WEIGHT	ADRENALS (2) MGM. /100 GRAMS BODY WEIGHT
			mgm. %	mgm. %	mgm. %		
Normal.....	12	154	68 \pm 2*	92	352	5.5	27.0
Normal given stilbestrol for 20 days.....	8	131	84 \pm 2	409	260	11	54.9
Normal given 2 stilbestrol pellets during 8 months.....	5	154	70 \pm 9	357	384	27.6	30.9
Thyroidectomized (8 months) given 2 stilbestrol pellets.....	5	166	69 \pm 9	288	415	17.0	17.0
Thyroidectomized (1 month).....	5	169	67 \pm 0.09	48	314	9	18.5
Thyroidectomized (1 month) given stilbestrol last 20 days...	8	162	66 \pm 5	354	313	11.9	21.0
Thyroidectomized (8 months)....	9	166	61 \pm 3	93	386	8.9	19.3
Thyroidectomized (8 months) given stilbestrol last 20 days...	10	168	76 \pm 2	671	411	13.1	22.3

* Standard error.

or 4 weeks, and then gained weight slowly. However, these animals failed to reach the weight of their litter-mate controls. This fact is not evident from the data in table 1, since the experiment was started on adult female rats picked at random.

Liver glycogen values in each group of rats given diethylstilbestrol were increased markedly (table 1) even though these animals ate less than the controls. This was particularly evident in animals which had been thyroidectomized for 8 months and given diethylstilbestrol for the last 20 days. The drug was not so effective in elevating liver glycogen when implanted in pellet form or when injected for 20 days into animals which had been thyroidectomized only 10 days prior to treatment. Thyroidectomy, per se, did not alter fasting liver glycogen values.

The muscle glycogen levels were not altered to any appreciable extent by estrogen treatment. The only value which varied greatly from the normal was the decreased muscle glycogen found in normal rats which were given diethylstilbestrol for 20 days (table 1). However, their smaller size could account, at least in part, for the decreased muscle glycogen values since it is more difficult to dissect and remove the tendons from the gastrocnemii in small animals.

Neither thyroidectomy nor diethylstilbestrol treatment caused much alteration of the blood sugar levels of rats fasted for 36 hours (table 1). Animals which had been thyroidectomized for 8 months receiving diethylstilbestrol for the last 20 days and normal animals which were given this drug for the same period of time, had only slightly higher blood sugar levels than the controls.

B. Urinary Nitrogen and Blood N.P.N. Urinary nitrogen determinations were made on normal rats which had received diethylstilbestrol for 20 days and on rats which had been thyroidectomized for 8 months and given diethylstilbes-

TABLE 2
Urinary nitrogen and blood N.P.N.

	NUMBER OF RATS	URINARY N KJELDAHL MGM./100 GRAMS BODY WEIGHT	URINARY N NESSLERIZATION MGM./100 GM. BODY WEIGHT	BLOOD N.P.N. mgm. %
Normal.....	12	87.1 \pm 2.7*		
Normal stilbestrol 20 days.....	8	104.2 \pm 5		
Thyroidectomized (8 months).....	6	97.3 \pm 7	94.7 \pm 7	51.5 \pm 2
Thyroidectomized (8 months) stil- bestrol last 20 days.....	6	76.3 \pm 3	75.0 \pm 3	39.0 \pm 2

* Standard error.

trol for the last 20 days. In the normal animals receiving this drug there was a slight rise in urinary nitrogen while in the thyroidectomized animals studied there was a slight reduction of urinary nitrogen (table 2). In order to check these last data, the urinary nitrogen was determined for some of the animals by both the micro-Kjeldahl and Nesslerization methods and at the end of the fast blood N.P.N. determinations were made. Data from these observations as shown on table 2 indicated: 1. There was no appreciable difference between the average total urinary nitrogen values, using either method, among animals of the same group. 2. There was an appreciable difference of total urinary nitrogen values and N.P.N. values between the two groups. 3. The ratio of N.P.N. and urinary nitrogen was very similar in each group.

C. Histology of Certain Organs. 1. The pituitary gland. The pituitary glands of rats which had been thyroidectomized and/or given diethylstilbestrol, were larger than the glands found in normal animals (see table 1). Pituitaries were the largest in animals which had received a prolonged stimulation of estrogen from diethylstilbestrol pellets. Glands of thyroidectomized rats given diethylstilbestrol for 20 days were larger than those of the thyroidectomized controls.

Increase in size of the pituitary glands in thyroidectomized rats was apparently caused by a hypertrophy of the basophils. The glands in animals which had received diethylstilbestrol exhibited degranulation of the chromophils, with a definite hyperplasia of the chromophobes. A resumé of the histological findings of the pituitaries in the various groups of animals is as follows: 1. The pituitaries of all thyroidectomized animals showed numerous "thyroidectomy" cells and the eosinophils were degranulated and very difficult or impossible to distinguish. 2. When diethylstilbestrol had been given to the thyroidectomized animals for 20 days, the "thyroidectomy" cells were absent or greatly reduced in number. 3. The pituitaries of normal animals having received diethylstilbestrol pellets for 8 months showed degranulation of the chromophils and a great increase in the number of chromophobes. For those rats which had been given the pellets at the time of thyroidectomy, "thyroidectomy" cells did not develop and the pituitaries of these animals were similar to those of normal animals which had received pellets.

2. Adrenals. In all thyroidectomized rats there was a reduction in the size of the adrenal glands. Treatment with diethylstilbestrol, however, caused a marked increase in the size of adrenals in normal animals, and a slight increase in the size of the adrenals in thyroidectomized animals (see table 1). Even with diethylstilbestrol therapy, however, the adrenals of thyroidectomized animals were smaller than those of normal controls.

The adrenal glands from normal animals, when fixed in Flemming's solution, showed a fairly definite pattern of lipid distribution. Abundant lipid was present as dense fine droplets of reduced osmic acid in the zona glomerulosa. The narrow subglomerular area was almost free of lipid. In the zona fasciculata it was rather dense and quite evenly distributed. The zona reticularis had a similar distribution, although certain cells were nearly filled with lipid material and had the appearance of being clumped.

When diethylstilbestrol was given to normal animals for 20 days there was a thickening of the adrenal cortex, especially in the zona fasciculata. The lipid was somewhat depleted after this treatment, particularly the lipid in the zonae fasciculata and reticularis.

Following thyroidectomy the whole cortex was reduced in thickness. This reduction was especially evident in the zona glomerulosa. The cells of the zona fasciculata, more often those in the outer part of the fasciculata, occasionally showed some vacuolation. The sinusoidal capillaries were wider than normally found in the inner part of the zona reticularis. The lipid distribution in these adrenals was quite similar to that found in adrenals of normal animals.

When diethylstilbestrol was administered for 20 days to thyroidectomized rats the lipid material of the adrenal cortex was reduced in amount. The glomerulosa still contained considerable lipid but there was a decrease in this fatty material in the zonae fasciculata and reticularis. Clumping of lipid was still evident to some extent in the zona reticularis and occasionally some clumping was found in the inner part of the zona fasciculata.

3. Liver. Histological studies were made to determine the distribution of lipid and glycogen in the liver from the various groups of rats and to observe whether the liver showed any evidence of pathology.

Liver glycogen was largely depleted in normal and thyroidectomized animals which had been fasted for 36 hours. The glycogen which was present was located in the cells adjacent to the central veins. The lipid, in relation to the glycogen, was more abundant in these animals and was located around the portal canal and extended out from it radially. There was considerably more lipid in the thyroidectomized animals than in the unoperated normal rats.

When diethylstilbestrol was administered to normal or thyroidectomized rats for 20 days, there was an increase in the amount of glycogen and a relative decrease in the amount of lipid as compared to the untreated animals. Some cells in the middle of the hepatic lobules contained both glycogen and lipid. As others have found, it was difficult quantitatively to estimate the amount of liver glycogen from histological preparations but these observations give some indication of the comparative glycogen content.

When pellets of diethylstilbestrol were implanted in normal or thyroidectomized animals both liver lipid and glycogen were present in quite large amounts. In one normal and in one thyroidectomized rat which had received pellets some hepatic cells showed fatty metamorphosis and a few others had undergone necrosis. Some sclerosis of the portal veins was also seen. Any pathology which was present, however, did not seem to be sufficient to impair liver function.

4. Ovaries. The histological structure of the ovaries was very similar in normal and thyroidectomized animals. The ovaries contained follicles in various stages of development, and mature, or involuting, corpora lutea. In normal rats injected with diethylstilbestrol for 20 days, the ovaries almost always contained large corpora lutea and small follicles. When pellets of diethylstilbestrol were implanted for a period of 8 months in normal or thyroidectomized rats, the ovaries were usually atrophic, exhibiting a few small follicles. When diethylstilbestrol was injected for 20 days into rats which had been thyroidectomized for 8 months, the ovaries of 12 out of 17 rats had follicular retention cysts. In addition to cysts, the ovaries always contained some large corpora lutea. Thyroidectomy, per se, or the injection of diethylstilbestrol into normal rats or rats which had been thyroidectomized for only one month did not cause the formation of cysts.

DISCUSSION. The administration of relatively large doses of diethylstilbestrol to normal rats or to rats which have been thyroidectomized for 8 months causes some rise in blood sugar levels. On the other hand, the blood sugar values for normal and thyroidectomized animals which received diethylstilbestrol pellets for 8 months and for rats which had been thyroidectomized for 1 month and given diethylstilbestrol do not vary appreciably from the normal. Earlier it was pointed out (1) that there was considerable rise in the blood sugar for 5 or 6 hours following the injection of diethylstilbestrol. However, this

acute effect was eliminated in the present studies because the animals were given their final injection of diethylstilbestrol 24 hours before blood samples were taken.

The blood sugar values for rats which were thyroidectomized for only 1 month are similar to those found in normal rats. However, there is a reduction in glyce-mic levels in animals which have been thyroidectomized 8 months. This latter datum is in accord with that of Bodansky (2), Moskalenko (3) and Sawada (4) who found that thyroidectomy in experimental animals resulted in a lowering of blood sugar values.

The most outstanding feature of the action of diethylstilbestrol on carbohydrate metabolism is its effect in elevating liver glycogen. Although it is not effective in elevating liver glycogen in adrenalectomized or hypophysectomized animals (5, 6), it does produce increased values in normals (7, 8), castrates (1) and as is evident in the present studies, in thyroidectomized animals. In fact, the liver glycogen values are higher in animals which have been thyroidectomized for 8 months and given diethylstilbestrol for 20 days than in any of the other animals examined. In accord with this, Van Horn (8) has shown that sex hormones are more effective when given to thyroidectomized animals. This fact seems to be verified in the present studies and probably results from the fact that the thyroidectomized animals, having a lower basal metabolic rate, do not conjugate or destroy the estrogens as rapidly in the liver as do normal animals.

The urinary nitrogen values, although somewhat variable, indicate that normal rats which have received diethylstilbestrol eliminate more urinary nitrogen than their controls. This increase in nitrogen excretion suggests the occurrence of gluconeogenesis from protein, as had previously been pointed out (7, 8). However, the rats which had been thyroidectomized for 8 months and then given diethylstilbestrol excrete less urinary nitrogen than thyroidectomized controls. The blood N.P.N. studies show a similar relationship. One should be cautious, however, in assuming that the changes in nitrogen excretion can be correlated with gluconeogenesis. Rats lose weight when they are given diethylstilbestrol and this fact alone could account for higher urinary nitrogen values in animals treated with this drug. Also, data represent the condition found at the termination of the experiment only and do not give a complete picture of nitrogen excretion during the entire experimental period. Nevertheless, since thyroidectomized rats receiving diethylstilbestrol excrete less nitrogen than the controls, it seems apparent that at least part of the increased liver glycogen comes from some source other than gluconeogenesis from protein.

The exact mechanism whereby diethylstilbestrol acts in increasing liver glycogen is not clearly understood. Previously it has been suggested that since diethylstilbestrol is not effective in elevating liver glycogen in adrenalectomized or hypophysectomized animals, its action in normal animals is an indirect one, working through one or both of these glands (5, 6). Recently, Ingle (10) has shown that diethylstilbestrol will cause some glycosuria in force-fed adrenalectomized-hypophysectomized-partially-pancreatectomized rats. Although this

suggests that diethylstilbestrol can modify carbohydrate metabolism in adrenalectomized-hypophysectomized animals to some extent, it seems likely that in elevating liver glycogen, this drug must act indirectly by way of the pituitary or adrenals, or through both glands.

The fact that thyroidectomy causes a vacuolation and clumping of the protoplasm of the basophils in the pituitary is well established (11, 12). This abnormal condition is largely corrected following 20 days of estrogen therapy, but in some pituitaries these "thyroidectomy" cells persist even after treatment. Indeed, there seems to be some correlation between the amount of liver glycogen and the condition of the basophils. In animals in which the "thyroidectomy" cells seem to be the least numerous the liver glycogen is the highest.

When normal animals are given diethylstilbestrol, the adrenals undergo considerable hypertrophy. If the diethylstilbestrol causes the adrenal to release more cortical hormone, which in turn causes an increase in the deposition of glycogen, as we believe it does, it seems logical that the animals with the largest adrenals should have the highest liver glycogen values. However, this does not hold true since the adrenals of rats which were thyroidectomized for 8 months and which had received diethylstilbestrol for 20 days had small adrenals, even smaller than those from normal controls. Thus, the size of the adrenal does not necessarily indicate the amount of its secretory activity, at least as far as the cortical hormones related to carbohydrate metabolism are concerned. In this regard, Ingle (13) produced a marked hypertrophy of the adrenal in diabetic rats by placing them under stress, but this did not alter their glycosuria.

SUMMARY

The effect of diethylstilbestrol on thyroidectomized rats has been studied. Rats were thyroidectomized for 1 month or 8 months and diethylstilbestrol was either injected during the last 20 days of the experiment or implanted as pellets for the entire 8 months.

A slight elevation in blood sugar was found following the administration of diethylstilbestrol for 20 days to both normal animals and animals which had been thyroidectomized for 8 months. Diethylstilbestrol, whether administered as pellets or injected into normal or thyroidectomized animals, caused a marked increase in liver glycogen. This increase was the greatest in the rats which had been thyroidectomized for 8 months and received diethylstilbestrol for 20 days. It was suggested that the estrogen is more effective in these rats because it is not destroyed or conjugated so rapidly in the liver.

When normal rats were given diethylstilbestrol for 20 days there was an increase in urinary nitrogen, suggesting that the increased liver glycogen was being formed by gluconeogenesis from protein. However, the urinary nitrogen values were reduced in rats which had been thyroidectomized for 8 months and given diethylstilbestrol for 20 days. This would indicate that the higher levels of glycogen in these animals was being derived, at least in part, from some source other than protein.

When diethylstilbestrol pellets were implanted in animals for 8 months the ovaries showed atrophic changes. After injecting diethylstilbestrol for a period of 20 days, the ovaries generally contained large corpora lutea and small follicles, except in the rats which had been thyroidectomized for 8 months, where a large proportion of the ovaries were cystic.

Assuming that diethylstilbestrol acts indirectly by way of the pituitary and adrenals in exerting its influence on carbohydrate metabolism, the size of the adrenals cannot be used as a criterion for an index of their secretory activity. The highest liver glycogen values were found in rats which had been thyroidectomized for 8 months and given diethylstilbestrol for 20 days, but their adrenals were smaller than those from normal animals.

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EFFECT OF COMPOUNDS RELATED TO GLYCOLYSIS IN MUSCLE ON THE SENSITIVITY OF MUSCLE TO ACETYLCHOLINE AND POTASSIUM¹

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During investigations of the behavior of striated muscle in patients with myasthenia gravis (1), the search for substances that modify the sensitivity of striated muscle to indirect and direct stimulation was undertaken. First the effect of substances that have been directly or indirectly related to the process of glycolysis in muscle were investigated with regard to their effect on the acetylcholine sensitivity of the rectus abdominis muscle of the frog. Secondly the effect of the substances on the potassium sensitivity of the striated muscle was also investigated to ascertain whether the changes of acetylcholine sensitivity of the muscle induced by the substances used are due to action on the special receptor mechanisms through which acetylcholine acts, or to changes of the sensitivity of the striated muscle to chemical stimuli in general.

EXPERIMENTAL. I. *Direct effect on the striated muscle.* The rectus abdominis muscle of the frog was excised and suspended in a muscle chamber containing 10 cc. Ringer's solution buffered by phosphate. The Ringer's solution was then changed for five minutes to one modified by inclusion of the particular substance under investigation. The muscle was then washed for ten minutes with Ringer's solution. This procedure was repeated increasing the concentration of the substances. The pH of all solutions used here, and in the following experiments, was corrected to 7. The amount of shortening of the muscle was registered by means of an isotonic lever on a kymograph. Whenever a shortening occurred as a result of immersion in one of the solutions the letter "s" was inserted in the table.

The results are given in table 1. Adenosinetriphosphate and hexosediphosphate induced a shortening of the rectus abdominis muscle. The electrical nature of the shortening induced by adenosinetriphosphate is described by Buchthal and Kahlson (2). The muscle shortening may be due either to removal of intracellular calcium by the phosphate liberated from the organic phosphates used, or to other mechanisms. Removal of calcium as the cause of muscle shortening is conceivable, since it is known that muscles and nerves with lowered calcium content show spontaneous activity. To ascertain whether removal of calcium would cause a shortening of the muscle, the effect of citrate, oxalate, fluoride, phosphate and immersion in calcium-free Ringer's solution on the muscle was also investigated. Immersion in phosphate and calcium-free

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Effect of the substances on the shortening of the rectus abdominis muscle induced by acetylcholine and potassium

SUBSTANCE	MAGNITUDE OF SHORTENING IN % OF CONTROL*. SHORTENING INDUCED BY:																		
	Acetylcholine										Potassium (20 mM)								
	(non-esterinized muscle) (30 μg)					(esterinized muscle) (8 μg)													
	Concentrations of the substances used (mg. in 100 cc. Ringer's solution):																		
	100	10	1	0.1	0.01	100	10	1	0.1	0.01	100	10	1	0.1	0.01	1000			
Hexosediphosphate.....	S†	204	155	112	103	S	205	147	115	104	S	157	122	112	102	103			
Dihydroxyacetone monophosphate.....			103	103	102	104						183	170	165	160	130			
β-Glycerophosphate.....	105	108	105	103	107						220	213	176	142	120				
Pyruvic acid.....	113	105	104	101	102						126	137	132	128	125	108			
Acetylphosphate.....	99	100	101	109	103						188	162	152	158					
Lactic acid.....	106	106	111	106	100						180	163	160	151	143	133			
Acetic acid.....	95	105	102	104	104						96	131	143	124	106	103			
Acetoacetic acid.....	101	105	104	105	103						131	153	147	137	125	120			
Acetone.....	139	117	102	105	100	106	98	100	103	97	106	96	109	104	100	98			
Acetaldehyde.....	129	102	100	97	100	70	92	98	103	100	85	82	131	130	100	100			
Ethylalcohol.....	102	104	106	107	107						98	100	100	104	98	101			
Butyric acid.....	75	97	101	103	96						124	140	143	123	100	99			
β-Hydroxy butyric acid.....	25	56	86	101	101						26	53	100	116	112	100			
Propionic acid.....	101	99	101	101	100						110	108	104	101	99	102			
Adenosinetriphosphate.....	S	244	155	115	101	S	287	164	105	99	S	158	121	107	100	100			
Creatinephosphate.....		138	117	97	99		130	118	103	100		109	109	102	109	102			
Creatine.....	103	104	99	103	105						126	120	113	117	113	113			
Creatinine.....	172	120	111	109	109	100	100	100	99	101	154	152	139	131	107	110			
Inosinic acid (muscle).....		146	121	108	103		156	119	103	101		135	140	132	127	105			
Ammonia.....	99	96	97	98	105						145	152	150	165	164				
Glyceraldehyde.....		100	101	99	96							105	105	99	100	98			
Fluoride.....	S	216	125	113	109	S	83	97	100	101	S	163	138	121	100	101			
Monoiodoacetate.....		108	98	103	102							100	99	105	105	100			
Adenosinetriphosphate in monoiodoacetate poisoned muscle† (Concentration of monoiodoacetate:																			
(1/250.000).....			193																
(1/25.000).....			175																
Adenosinetriphosphate in fluoride poisoned muscle† (Concentration of fluoride:																			
3 mgm. per 100 cc.).....			191																
Pyrophosphate.....	102	94	95	101	104						197	131	116	111	109	105			
Citrate.....	S	110	106	103	102						S	166	145	133	131	112			
Oxalate.....	S	101	102	103	99						S	161	141	134	120				
Phosphate.....	104	106	102	102	100						152	144	130	124	117				
Epinephrine.....			137	116	98			130	120	100			58	66	83				
Physostigmine.....			388	195	125						153	132	135	131	130	100			

* Each value represents the average of 10 separate experiments. The S.E. of the mean for each value was less than $\pm 4\%$.

† "S" means shortening of muscle occurred during the immersion of the muscle in the solution for 5 minutes.

‡ Muscle immersed for 1 hour in the monoiodoacetate and fluoride solution before the experiment with adenosinetriphosphate and acetylcholine.

Ringer's solution did not induce shortening of the muscle, probably because the loss of the intracellular calcium ion by these two measures was not large enough (3). Shortening of muscle occurred in the presence of citrate, oxalate, and fluoride. Similar amounts of fluoride were required to induce a shortening of both untreated and eserinated muscles, suggesting that the shortening of the muscle was not due to the ability of fluoride in inhibiting cholinesterase.

II. *Effect on the acetylcholine sensitivity of muscle.* The rectus abdominis muscle was prepared as described above. Shortening of the muscle was induced throughout the entire experimental procedure by immersion in an acetylcholine solution (50 μ g per 100 cc. Ringer's solution) for two minutes. Between two immersions in acetylcholine the muscle was washed with Ringer's solution for ten minutes. This procedure was repeated until three successive immersions in the solution of acetylcholine gave similar responses. The muscle was then washed for five minutes and immersed in one of the solutions of the substances for five minutes. A series of solutions containing the substances in increasing concentrations was used. The amount of muscle shortening was registered by means of an isotonic lever on a kymograph and the tracings were measured. The amount of shortening of the muscle after immersion in the solutions of the substances was expressed as percentage of the amount of shortening of the same muscle before immersion in the solutions. The amount of shortening induced by acetylcholine in control muscles immersed only in Ringer's solution remained unchanged for at least three hours. This period of time was longer than the duration of the experiments described. Since the S.E. of the mean for each experiment was less than ± 4 per cent, all results deviating from 100 per cent by more than 12 per cent are probably significant deviations.

$$(2\sqrt{\text{S.E.}_{(\text{control})}^2} + \text{S.E.}_{(\text{experiments})}^2) = \sqrt{2 \times 4^2 + 4^2} = 11.5).$$

The acetylcholine sensitivity of the muscle was increased by hexosediphosphate, adenosinetriphosphate, creatinephosphate, creatinine, inosinic acid, epinephrine, physostigmine, fluoride, acetone, and acetaldehyde. The acetylcholine sensitivity of the muscle was decreased by β -hydroxybutyric acid and by butyric acid (higher concentrations); and was not modified by the other substances used.

To ascertain whether the acetylcholine sensitivity of the muscle increased by the same mechanism involved in the increase of acetylcholine sensitivity caused by physostigmine, muscles were immersed for one hour in a Ringer's solution containing 2.5 mgm. physostigmine salicylate per 100 cc. The substances were dissolved in physostigmine solutions and the experiments were carried out as described above, except that the physostigmine solutions were used instead of Ringer's solutions. Shortening of muscle was induced by a solution containing 8 μ g acetylcholine per 100 cc. Ringer's solution. (The physostigmine solution was concentrated enough to cause nearly maximal sensitization of the muscle to acetylcholine.)

The results are given in table 1. Hexosediphosphate, adenosinetriphosphate, inosinic acid and epinephrine increased the acetylcholine sensitivity of untreated

and eserinizd muscles to a similar extent. Therefore, these substances increased the acetylcholine sensitivity by other mechanisms than does physostigmine.

The increase of acetylcholine sensitivity by the above enumerated substances was not due to metabolites of hexose, since either immersion in metabolites of hexosediphosphate, or poisoning by monoiodoacetate and glyceraldehyde did not modify the acetylcholine sensitivity of the muscle (table 1).

The increase of acetylcholine sensitivity of the striated muscle in the presence of physostigmine, fluoride, acetone, acetaldehyde, and creatinine was mainly due to the effect of these substances in inhibiting cholinesterase (4), since these substances did not increase the acetylcholine sensitivity of eserinizd muscle.

Effect of removal of calcium. To ascertain whether the substances used exerted their effect on the acetylcholine sensitivity of the muscle by removing some of the muscle calcium ions, the following experiments were performed: muscles were immersed in citrate, oxalate, phosphate, and calcium-free Ringer's solution. (The pH of all solutions used was corrected to 7.) The acetylcholine sensitivity of the muscle was not modified by the first three substances (tables 1, 2), and was decreased by immersion in calcium-free Ringer's solution.

A slight increase of the calcium content of the muscle induced by immersion in a 9 mM CaCl_2 solution did not modify the acetylcholine sensitivity of the muscle (5) (table 2).

These results suggest that any increase of acetylcholine sensitivity observed in the above experiments was not due to a decrease of the calcium ion content of the muscle.

Effect of the substances on acetylcholine synthesis within the rectus abdominis muscle preparation. Since the excised muscle contains nerve endings, it was necessary to ascertain whether the muscle shortening and increased acetylcholine sensitivity observed were due to an increase of acetylcholine synthesis inside the nerves as a result of the action of the substances used. This is pertinent since some of the substances used are known to increase acetylcholine synthesis (6, 7, 8). It is improbable, however, that the results reported were due to increased acetylcholine synthesis because of the following observation: muscles were treated as described above. Shortening of the muscle was induced by a 2 mgm. per 100 cc. acetylcholine solution instead of a 50 μg per 100 cc. solution. Further increase of the concentration of acetylcholine did not induce a greater muscle shortening. The acetylcholine sensitivity of these muscles was, however, increased by immersion in an adenosinetriphosphate solution (1 mgm. per 100 cc. Ringer's solution) for five minutes before immersion in the acetylcholine solution. This increase was 60 per cent ± 2.0 (average of 10 expts.). Therefore, the increase of acetylcholine sensitivity in the above experiments could not be due to an increase of the local concentration of acetylcholine due to increased synthesis.

III. *Effect of the substances on the potassium sensitivity of muscle.* To ascertain whether the substances modify the sensitivity of the striated muscle to other chemical stimuli, muscle shortening was induced by a 20 mM KCl solution in-

stead of acetylcholine, and the effect of the substances on the potassium sensitivity of the muscle was observed as described above. Shortening of muscle induced by potassium was frequently described, but the mechanism of muscle shortening induced by potassium is not yet known. Acetylcholine and potassium induce different physicochemical changes in the muscle cell (9-13) and even different physical changes in isolated myosin (14). Acetylcholine sensitivity usually parallels the sensitivity of the muscle to indirect stimulation; the potassium sensitivity often parallels the effect of direct stimulation of the muscle (15).

TABLE 2

Effect of changes in calcium and potassium ion content of muscle on acetylcholine and potassium sensitivity of muscle

MUSCLE IMMERSSED IN:	MAGNITUDE OF SHORTENING IN % OF CONTROL* (Shortening induced with acetylcholine (50 µg per 100 cc.))										
	Time of shortening after beginning of immersion in minutes:										
	10	20	30	40	50	60	70	80	90	100	
Calcium-free Ringer's solution	101	97	95	96	96	91	89	84	81	76	
Ringer's solution containing 9 mM CaCl ₂	98	97	94	94	92	91	93	92	91	90	
Potassium-free Ringer's solution	104	103	99	95	94	91	90	97	82	79	
Ringer's solution containing:											
10 mM KCl	100	97	98	96	97	96	95	93	94	92	
6 mM KCl	103	100	100	97	98	101	100	98	97	96	
4 mM KCl	103	98	96	95	101	100	97	96	95	95	
	(Shortening induced with potassium (20 mM KCl))										
Calcium-free Ringer's solution	108	125	132	145	156	175	196	217	234	261	
Ringer's solution containing 9 mM CaCl ₂	74	73	71	70	68	66	63	62	61	60	
Potassium-free Ringer's solution										55	
Ringer's solution containing:											
10 mM KCl	118	132	144	166	150	136	125	116	104	104	
6 mM KCl	102	116	132	148	157	160					
4 mM KCl	125	126	124	138	134	142	146	147	155	168	

* Each value represents the average of 10 separate experiments. The S.E. of the mean for each value was less than $\pm 4\%$.

The results are given in tables 1, 2. The potassium sensitivity of the muscle was increased by most of the substances used, was not modified by acetaldehyde, creatinephosphate, and monoiodoacetate, and was decreased in the presence of Ringer's solution with high calcium content, potassium-free Ringer's solution, epinephrine in all concentrations used, and inosinic acid, acetaldehyde, β -hydroxybutyric acid in higher concentrations.

The experiments add the following information about the mechanism of shortening of muscle induced by potassium: 1, measures inducing a decrease of the calcium ion content of muscle (oxalate, citrate, fluoride, immersion in calcium-free Ringer's solution, Ringer's solution containing an increased amount of

potassium) increased the potassium sensitivity of the muscle, and 2, many metabolites of hexosediphosphate increased the potassium sensitivity of the muscle. The potassium sensitivity of the untreated muscle as tested above, however, does not depend on the glycolysis occurring in the muscle, since poisoning with monoiodoacetate and glyceraldehyde did not decrease the potassium sensitivity of the muscle.

Effect of the activity of —SH group on acetylcholine and potassium sensitivity of muscle. To ascertain whether active —SH groups are involved in changes of acetylcholine and potassium sensitivity of the muscle, further experiments with monoiodoacetate and pyrophosphate were performed. It was observed that monoiodoacetate, a potent inactivator of the —SH group, did not modify the acetylcholine and potassium sensitivity of the muscle (table 1). Pyrophosphate, a substance known to protect, in low concentrations, the —SH group from oxidation (16, 17), and, in higher concentrations, to inhibit various enzyme systems (18–20), did not modify the acetylcholine sensitivity of the muscle, and increased the potassium sensitivity (table 1). Therefore, it seems that there is no simple relationship between activity of the —SH groups and changes of acetylcholine and potassium sensitivity of the muscle.

DISCUSSION. Based on the work of Engelhardt and collaborators (21), Needham and collaborators (14), and Sandow (22) it is currently held that: when a mechano-chemical coupling of myosin and adenosinetriphosphate in the form of an enzyme-substrate combination occurs, energy is provided for some contractile mechanism of the muscle cell.

In the above described experiments, immersion in solutions of adenosinetriphosphate induced a shortening of the muscle and increased the acetylcholine sensitivity (table 1). Adenosinetriphosphate had a more marked effect on the muscle (table 1) when dephosphorylation was partially prevented during penetration into the muscle, e.g., by poisoning the muscle with large amounts of fluoride (23). Hexosediphosphate and creatinephosphate probably exerted their effect by promoting adenosinetriphosphate formation (24), since myosin has a great specificity for adenosinetriphosphate and does not dephosphorylate hexosediphosphate and creatinephosphate (14, 25).

The effect of adenosinetriphosphate in inducing muscle shortening and in increasing acetylcholine sensitivity was not due to: 1, increased acetylcholine synthesis; 2, inhibition of cholinesterase (since adenosinetriphosphate increased the acetylcholine sensitivity of eserinizied and untreated muscles to a similar extent); 3, increased glycolysis (since adenosinetriphosphate increased the acetylcholine sensitivity of untreated muscles and muscles poisoned with monoiodoacetate to a similar extent). Removal of calcium by adenosinetriphosphate did not contribute to the increase of acetylcholine sensitivity; removal of calcium by adenosinetriphosphate may have contributed to muscle shortening induced by adenosinetriphosphate.

It is suggested that the mechanism involved in both increase of acetylcholine sensitivity and induction of muscle shortening by adenosinetriphosphate is the same, namely, a union of myosin and adenosinetriphosphate that liberates

energy for a contractile mechanism. Further, the data are compatible with the postulate that changes in amount or distribution of intracellular adenosinetriphosphate is an important factor in the initiation of muscle contraction.

SUMMARY

1. The effect of products of glycolysis, substances involved in the esterification of carbohydrates, and substances inhibiting glycolysis (a) on the rectus abdominis muscle of the frog, and (b) on the acetylcholine and (c) potassium sensitivity of this muscle, was investigated.

2. Hexosediphosphate, adenosinetriphosphate, fluoride, citrate, and oxalate in relatively high concentrations induced shortening of the muscle in the absence of any other agent.

3. Hexosediphosphate, pyruvic acid, acetone, acetaldehyde, adenosinetriphosphate, creatinephosphate, creatinine, inosinic acid, epinephrine, physostigmine, and fluoride increased the acetylcholine sensitivity of the muscle.

4. Dihydroxyacetone monophosphate, β -glycerophosphate, acetylphosphate, propionic acid, acetic acid, acetoacetate, ethyl alcohol, creatine, ammonia, citrate, oxalate, phosphate, pyrophosphate, glyceraldehyde, and monoiodoacetate did not modify the acetylcholine sensitivity.

5. Butyric acid (high concentrations) and β -hydroxybutyric acid decreased the acetylcholine sensitivity of the muscle.

6. Potassium-free Ringer's solution, Ringer's solution with high calcium content, epinephrine, in biological concentrations, acetaldehyde and β -hydroxybutyric acid in higher concentrations decreased the potassium sensitivity of the muscle.

7. Propionic acid, acetone, ethyl alcohol, creatinephosphate, glyceraldehyde, and monoiodoacetate did not modify the potassium sensitivity of the muscle; all the other substances used increased it.

8. There seems to be no direct relationship between activity of —SH groups and changes in acetylcholine and potassium sensitivity of the muscle.

9. It is inferred from the above experiments that: (a) measures lowering the calcium ion content of the muscle increase the potassium sensitivity and induce shortening of the muscle; (b) changes in distribution or in increase of intracellular adenosinetriphosphate increase the acetylcholine sensitivity of the muscle. The data are compatible with the postulate that changes in amount or distribution of intracellular adenosinetriphosphate is an important factor in the initiation of muscle contraction.

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ARTERIAL OXYHEMOGLOBIN SATURATIONS AT CRITICAL PRESSURE-ALTITUDES BREATHING VARIOUS MIXTURES OF OXYGEN AND NITROGEN: WITH A NOTE ON THE EFFECT OF EXERCISE¹

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The relationship between the oxygen tension of inspired air and the oxyhemoglobin saturation of the arterial blood has assumed importance in aviation medicine because oxygen equipment which delivers inspiratory gas mixtures to the aviator is judged from the physiological point of view by its ability to maintain an adequate oxyhemoglobin saturation of the arterial blood. Dill has examined this relationship in subjects breathing air up to an altitude of 20,000 feet and breathing pure oxygen up to an altitude of 45,000 feet (2). His observations have been confirmed by many investigators (5), and have proved invaluable in establishing physiological criteria for the design of present day oxygen regulators.

In actual practice the aviator may be exposed at critical altitudes to breathing mixtures which contain appreciable amounts of nitrogen. This nitrogen dilution may result from inadvertent mask leak, impurities in the supply of breathing oxygen, or the failure of the diluter-demand oxygen regulator to provide pure oxygen at the prescribed pressure-altitude. Experimental data are required, therefore, to define the effect of various oxygen-nitrogen mixtures at critical altitudes on the oxyhemoglobin saturation of arterial blood. Wulff described such experiments and defined the saturations (measured by the Millikan oximeter) that obtain at 25, 30 and 35 thousand feet (15).

Microgasometric methods devised by Scholander and Roughton provided the means for determining the oxyhemoglobin saturation and the CO₂ content of small samples of blood, and recent studies in this laboratory confirmed the earlier finding that cutaneous blood can be arterialized by applying heat (8, 9). In the present study microgasometric analyses of blood from the heated ear lobe were used to estimate the arterial oxyhemoglobin saturations which obtain in man when breathing various oxygen-nitrogen mixtures at critical pressure-altitudes in the decompression chamber. The CO₂ content was used as a measure of the degree of acapnia and to estimate the magnitude of the Bohr effect. In addition, certain observations were made of the effect of exercise on arterial oxyhemoglobin saturation at altitude during moderate anoxia.

¹ The opinions or assertions contained herein are the private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

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METHODS. The 24 subjects consisted of 16 males and 8 females ranging in age from 19 to 34 years. No subject was studied more than once at any given altitude or oxygen-nitrogen mixture. The exercise experiments were performed on the male subjects only.

The analytical methods have been described in detail elsewhere; the micro-gasometric methods of Roughton and Scholander (13) were used for determinations of the O_2 saturations and CO_2 contents of samples of cutaneous blood obtained from the ear lobe which had been vasodilated by heat. Analyses of such samples have been shown to yield accurate estimations of the state of the arterial blood (8, 9). The oxygen content of the inspired breathing mixtures

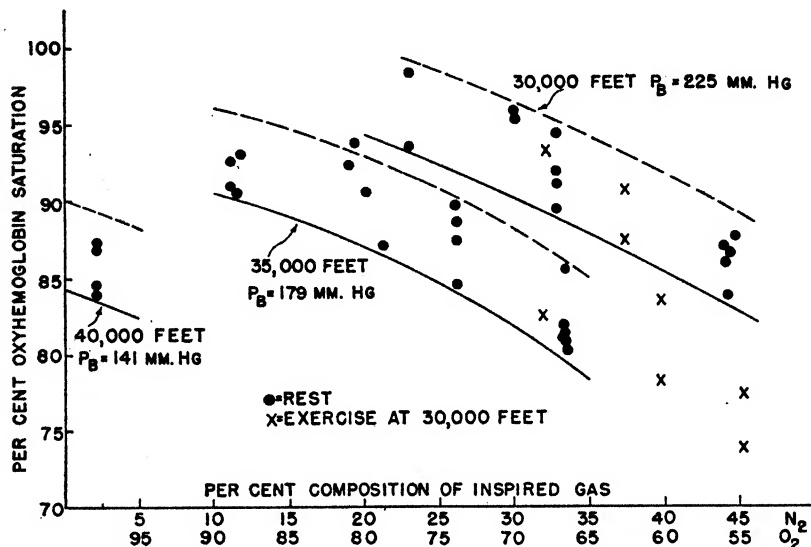


Fig. 1. Oxyhemoglobin saturation of "arterial" blood related to altitude and composition of inspired gas. The range for a given altitude is defined by the area within a 6 per cent limit by the continuous and interrupted curves.

was determined at sea level and at altitude by the Scholander nitrogen analyzer (14) which was calibrated empirically for the effects of reduced barometric pressure (10).

The experiments were carried out in a decompression chamber. Decompression to the desired pressure-altitude was accomplished at the rate of 5,000 feet per minute while the subjects breathed from a demand oxygen regulator. The test breathing mixtures were made up of suitable proportions of oxygen and nitrogen in high pressure tanks. The gas was delivered to the subject by means of a mouth-piece to avoid any dilution from ambient air. The experiments at rest were performed in pairs with the two subjects breathing from the same tank through the same regulator with appropriate check valves to prevent re-breathing.

The experiments at rest were executed while the subjects sat quietly. During exercise, the work was accomplished and graded on a bicycle ergometer. The degree of work varied among the experiments from 500 to 755 kgm. m./min. (3630 to 5480 ft. lbs./min.)

During the first hour of exposure to reduced oxygen tension progressive changes occur in the respiratory quotient; i.e., alveolar equilibrium or a respiratory steady state is not attained until 45 minutes or more have elapsed (11). Exposures of this duration were not practicable in this study; in the experiments reported here the samples of blood were taken 10 to 12 minutes after the subject had been breathing the required mixture at the test altitude. In the experiments with exercise the subjects sat at rest for 5 minutes and then exercised for 5 minutes at the close of which period the sample of blood was taken.

RESULTS. The results of these studies are presented in the accompanying figure. The range of oxygen saturation among the subjects did not exceed 6 per cent for a given altitude and nitrogen dilution. The heavy lines in the figure indicate the lower limit of the range of saturations for each pressure-altitude studied. These lines, therefore, represent the expected minimal saturations at rest for a given altitude and nitrogen dilution. These limits may be used to predict the maximal allowable nitrogen dilution at a given altitude compatible with a given arterial oxyhemoglobin saturation. For example:

	Altitude	Inspired gas		
		O ₂ per cent	N ₂ per cent	per cent "leak"
To maintain a saturation of 90 per cent	30,000	70	30	37
	35,000	88	12	15
To maintain a saturation of 85 per cent	30,000	60	40	50
	35,000	75	25	31
	40,000	100	0	0

Per cent "leak" expresses the contribution from ambient air to the breathing mixture necessary to produce a given nitrogen dilution.

The CO₂ contents of the "arterial" blood were found to range within relatively close limits. The average values (and the extremes) are presented below:

Altitude	CO ₂ vols per cent
30,000 (rest)	47.6 (42.6-50.8)
30,000 (exercise)	42.0 (39.1-44.7)
35,000 (rest)	46.2 (43.7-48.6)
40,000 (rest)	43.1 (41.3-46.2)

There was noted a rough inverse relation between the CO₂ contents and the oxygen saturations (Bohr effect); but the variations in oxyhemoglobin saturations among the various subjects cannot be attributed solely to the differences in arterial CO₂ contents.

DISCUSSION. The data obtained in this study indicate that the theoretical predictions of oxyhemoglobin saturations by the various "alveolar air" formulae are sound when the subject breathes various oxygen tensions at altitude at rest

(6). The average oxyhemoglobin saturations determined by Wulff by the Millikan oximeter likewise correspond to the values obtained here (15). In agreement with Wulff we are inclined to attribute the wide variations observed by him both to the technical difficulties of oximeter determinations and to the Bohr effect resulting from hyperventilation.

The lowering of arterial oxyhemoglobin saturation by exercise in moderately anoxic states has been described by Barcroft and has been observed by other investigators (1, 4, 7). In this laboratory, data from other studies have shown that the arterial oxygen *tension* falls during moderate exercise (3, 12). This fall in arterial oxygen tension is compatible with the decrease in arterial oxyhemoglobin saturation.

There are certain practical implications inherent in the data reported in this study:

a. Reference to the figure indicates that at pressure-altitudes up to 30,000 feet relatively large mask leaks will be tolerated. For example, at 30,000 feet 37 per cent of the inspired gas may be contributed from ambient air and the minimal oxyhemoglobin saturation will still exceed 90; at 35,000 feet 15 per cent of the inspired gas may be ambient air and the oxyhemoglobin saturation will not fall below 90.

b. The blood of subjects who are mildly anoxic may become further unsaturated with respect to oxygen during a brief period of moderate to heavy work. This possibility suggests again that at altitudes above 25,000 feet it is advisable to provide inspired gas mixtures with oxygen tensions that approach sea level equivalents.

SUMMARY

Microgasometric methods for analysis of "capillary" blood samples have been applied to a study of the arterial oxyhemoglobin saturations which obtain in man when breathing various oxygen-nitrogen mixtures at critical pressure-altitudes in the decompression chamber. Forty-three observations on 24 subjects at 30, 35 and 40 thousand feet established the following relationships:

1. The arterial oxyhemoglobin saturation may be predicted with an accuracy of ± 3 per cent for a given oxygen-nitrogen mixture at these pressure-altitudes when the subject is at rest.

2. The arterial oxyhemoglobin saturation may fall appreciably during a short period of moderate to heavy work in states of mild anoxia.

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THE INFLUENCE OF AVOIDANCE CONDITIONING ON THE COURSE OF NON-AVOIDANCE CONDITIONING IN DOGS

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Whether the avoidance conditioning procedure or the non-avoidance conditioning procedure is more effective in the development of conditioned responses has great practical and theoretical importance. In both of these types of training a noxious unconditioned stimulus is used to elicit the response, but in the avoidance type the animal can avoid the unconditioned stimulus by responding correctly to conditioned stimulus whereas in the non-avoidance type the unconditioned stimulus is applied as a "reinforcement" even though the animal responds correctly to the conditioned stimulus.

There is a controversy in the literature as to which procedure is more effective, some workers finding no difference, others reporting avoidance training to be superior, and still others finding non-avoidance training to be better. All experiments so far performed have involved statistical group comparisons in rats and guinea pigs (Schlosberg, 1934, 1936; Hunter, 1935; Schlosberg and Kappauf, 1935; Brogden, Lipman, and Culler, 1938; Munn, 1939; Sheffield, 1941).

The experiments to be described in this paper were designed to overcome some of the difficulties involved in the use of rats and guinea pigs by using the leg-flexion response in dogs, and to eliminate the factor of animal variation inherent in group comparisons by eliciting both avoidance and non-avoidance responses concurrently in each of the animals.

PROCEDURE AND RESULTS. In the non-avoidance conditioning the duration of the conditioned stimulus was three seconds, during the last one second of which an electrical stimulus to the paw (unconditioned stimulus) was applied. In the avoidance conditioning the procedure was the same except that if the animal flexed the correct leg within two seconds, the conditioned stimulus was turned off and the animal did not receive the electrical stimulus.

Four series of tests were carried out on three dogs. The first dog was conditioned in the avoidance fashion to flex its left foreleg to the sound of a bell and, in non-avoidance fashion, to flex its right foreleg to a loudspeaker hum. The curve of performance for each leg is shown in figure 1A. Avoidance and non-avoidance sessions were alternated, only one being run per day, and the sessions are represented in pairs on the curve. The curve of the "avoidance" leg leveled off at approximately 95 per cent correct responses whereas that for the "non-avoidance" leg, after marked fluctuation, came down to a level of approximately 20 per cent. The training procedure was then reversed: the original conditioned stimulus for the right leg was retained, but the response was treated in the avoidance manner; the original conditioned stimulus for the left leg was likewise retained, but the response was treated in the non-avoidance fashion. The

superior performance of the "avoidance" leg was again very definite as shown on the curve. There are two very important features of this experiment which are not shown by the curves: 1. In the "non-avoidance" sessions in which the percentage of responses was very low, it was not absence of response which brought it so low, but rather a flexion of the "avoidance" leg instead of the correct leg. 2. During "non-avoidance" sessions the animal was very restless, but during "avoidance" sessions it was calm.

The next part of the experiment consisted in subjecting the poorly performing "non-avoidance" leg (left leg at this stage) to consecutive "non-avoidance"

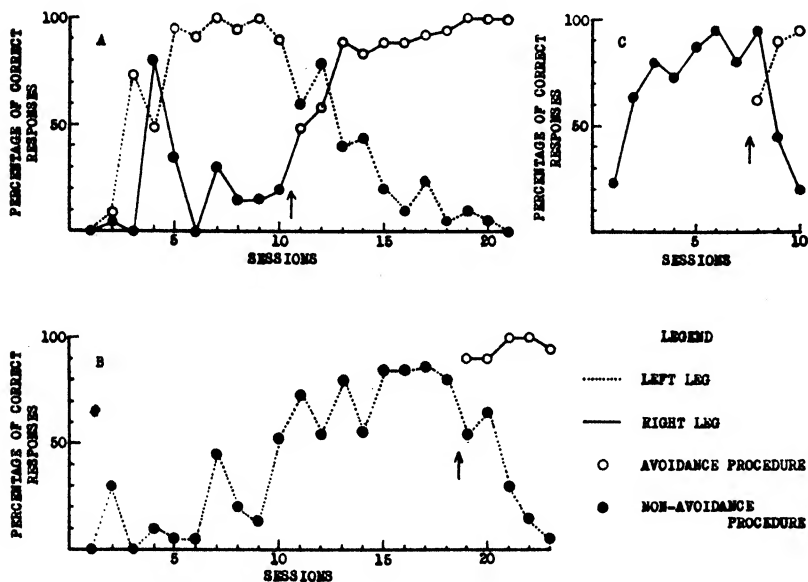


Fig. 1. The curves of performance of "avoidance" and "non-avoidance" legs under various experimental conditions. A. "Avoidance" and "non-avoidance" sessions were alternated daily right from the start of the experiment and procedures for the legs were reversed at the arrow. B. The poorly performing "non-avoidance" leg at the end of curve A was subjected to consecutive "non-avoidance" sessions and then the "avoidance" leg re-introduced at the arrow. C. Procedure was the same as that for curve B except that the dog had not been previously conditioned.

sessions, there being no "avoidance" sessions to interfere with performance, letting the animal reach a high level of performance, and then reintroducing the "avoidance" sessions of the opposite leg in alternation with the "non-avoidance" sessions. The results are shown in the curve of figure 1B. There are two important features which are likewise not shown by the curve: 1. During the eighteen day period in which only non-avoidance conditioning was being run, the animal became very calm and lost all the restlessness which previously had characterized the "non-avoidance" sessions. 2. When the "avoidance" sessions were again introduced, restlessness reappeared in the "non-avoidance" sessions.

The results obtained with dogs 2 and 3 were simply confirmations of the performance of the dog just described. The experiment of figure 1A was repeated on dog 2. Since the results agree with those of dog 1 in all respects, the curves of performance are not included here. The experiment of figure 1B was repeated on dog 3 except that this dog had not been previously conditioned in any manner whatsoever. The results are shown in figure 1C.

The results of these experiments are so clear-cut that no statistical treatment is necessary.

DISCUSSION. Non-avoidance conditioning when carried out alone in dogs can maintain a high level of performance, and the animals remain calm during the training procedure. In our laboratory we have maintained performance at 100 per cent correct responses through the use of this procedure. Avoidance conditioning when carried out alone in dogs will also maintain a high level of performance. When both procedures are carried out concurrently in the same dog, the performance of the "avoidance" leg reaches its customary level, but that of the "non-avoidance" leg becomes very poor. Not only does the "avoidance" leg reach a higher percentage of correct responses but also the animal responds with its "avoidance" leg to the "non-avoidance" conditioned stimulus a good percentage of the time. In fact this was the main cause of the poor performance in non-avoidance training when it was carried out concurrently with avoidance training.

In this situation a conflict is developed between a biologically useful response (lifting of the paw to avoid a noxious stimulus) and the biologically useless response represented by the non-avoidance type of leg-flexion. The fact that the latter can be developed and maintained with ease when tested alone indicates the ability of the nervous system to yield conditioned activities of no value to the organism; but its inability to handle well a useless activity concurrently with a useful activity is demonstrated in the prepotence which the avoidance response acquires over the non-avoidance response. The conflict between the two responses existing during the "non-avoidance" sessions may account for the restlessness which occurred only during the "non-avoidance" sessions.

The pathways opened by the avoidance procedure seem to have such a low resistance, or such a low threshold, as compared to those opened by the non-avoidance conditioning procedure, that the impulses set up by the "non-avoidance" conditioned stimulus follow the "avoidance" pathways in spite of the training to which the animal is subjected. The impulses set up by two auditory stimuli which differ markedly in pitch and quality probably project upon the cerebral cortex at different spots, but cortical neurons are so intricately connected with one another that it would be very possible for impulses arriving at one spot to activate neurons associated with another spot especially when these other neurons have acquired a very low threshold as a result of the conditioning process.

We find it very difficult at the present time to explain why the avoidance conditioning procedure should work so well at lowering pathway resistances. Some type of reinforcing agent is necessary to maintain performance in the usual type of conditioning, but in avoidance conditioning we can find no such reinforce-

ment. A possible explanation is that the dog is capable of some crude sort of thinking which enables it to realize that by lifting its paw it avoids an unpleasant sensation. It must be aware of the existence of something that it is not experiencing. That the thinking process is capable of supplying the reinforcement necessary to maintain a conditioned response is shown by its ability to do so in the avoidance conditioning of human beings.

CONCLUSIONS

Whereas the avoidance type and the non-avoidance type of conditioning procedure each by itself can develop and maintain conditioned leg-flexion performance in dogs at a high level, when these two types of conditioning are carried out concurrently in the same animal the avoidance conditioning has a marked deleterious effect on the course of the non-avoidance conditioning but is itself fully retained.

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A STUDY OF THE WATER LOSSES THROUGH THE SKIN IN THE RAT

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An estimate of the amount of water lost by an animal through the respiratory tract can be calculated from the respiratory volume, determined from oxygen consumption data, if the relative humidity in the apparatus during the experiment is known. During a series of measurements with rats in an open circuit apparatus in this laboratory, it was found that this estimated value accounted for less than half of the total insensible water loss, determined by drying the air stream after it had left the animal chamber. Calculations made on the basis of the results of Greene and Luce (1), Greene (2), Swift and Forbes (3) and Black and Swift (4) showed again that this was true, and it thus appeared that considerable amounts of water must come from the skin of the rat in spite of the fact that the sweat glands of the rat have been reported to be rudimentary and non-functional.

In human subjects the amount of water lost through the skin has been reported by several observers (5, 6, 7, 8) to be from 50 to 80 per cent of the total insensible loss, but no measurements of this type in rats have appeared in the literature. For this reason the experiments reported here were undertaken; the amounts of water lost insensibly through the skin and from the respiratory tract of the rat have been determined under normal environmental conditions, and under conditions approximating those of the tropics.

EXPERIMENTAL. The measurements were carried out on one animal at a time in an air conditioned room using a chamber five and one-half inches in diameter with a volume of four and one-half liters. A floor of one-half inch wire mesh supported the rat, which was anesthetized during the first experiments, and a pool of mineral oil in the bottom of the apparatus prevented the evaporation of water from urine and feces. To determine the water loss through the skin, room air was drawn at a rate of 1000 ml. per minute by means of a suction pump into the opening at the top of the chamber, around the body of the rat, out at the bottom of the chamber, through weighed drying tubes, and through a gas meter.

In order to separate the moisture contained in the expired air from that lost through evaporation from the skin, the head of the rat was pushed into a hole in a rubber diaphragm which was stretched over a piece of glass tubing 75 mm. long and 42 mm. in diameter, until the nose and mouth were completely inside. The other end of the tubing was closed with a two-hole rubber stopper and was connected with the outside of the chamber by means of two glass tubes. To determine the water loss from the respiratory tract, room air was drawn at a rate of 125 ml. per minute around the nose and mouth of the rat and through weighed drying tubes by means of a calibrated siphon bottle.

Blank determinations of the water content of the room air were made during each experiment. The amounts of water lost from the skin and from the respiratory tract were calculated from the weight gains in the drying tubes by subtracting the weight of water originally in the same volume of room air, as determined in the blank.

A modified form of the apparatus was used for the later experiments in which the rats were not anesthetized, but were confined in a wire mesh tube fastened to the supporting floor. The air entering the apparatus was pumped through sulfuric acid, 43 per cent by weight, at a temperature of 23.3°C. to bring it to

TABLE 1
Water loss from the skin and from the lungs of the rat

RAT	WEIGHT	EXPIRED WATER PER 30 MIN.	WATER EVAPORATED FROM SKIN PER 30 MIN.	SKIN EVAPORATION AS PER CENT OF TOTAL
Group I—Anesthetized rats—Intake air 22.4 to 23.9°, Relative humidity 47-58%				
	<i>grams</i>	<i>mgm.</i>	<i>mgm.</i>	
1	324	22	51	70
2	327	30	112	79
3	382	39	88	69
4	278	25	52	68
5	374	24	86	78
Group II—Anesthetized rats—Intake air 33.2 to 34.2°, Relative humidity 48-55%				
1	312	59	144	63
2	350	49	115	70
3	282	46	130	74
4	332	45	113	72
5	344	44	177	80
Group III—Unanesthetized rats—Intake air 23.3 to 25.3°, Relative humidity 46-50%				
1	349	72	103	59
2	317	69	100	59
3	372	115	112	49
4	344	94	157	63
5	397	95	125	57

constant humidity, and then into the chamber. After passing over the body of the rat the greater part of the air flowed out of the chamber, through drying tubes, and through a gas meter at a rate of 1200 ml. per minute, and the remainder of the chamber air was drawn around the nose and mouth of the rat, through drying tubes and through a gas meter at a rate of 500 ml. per minute by means of a suction pump.

With this form of the apparatus blank determinations were made alternately with the measurements on the animals, and during these, a tightly capped bottle of warm water was placed in the chamber to bring it to the same temperature that existed when the chamber housed a rat. The increased concentration of

water in the air which passed around the nose and mouth of the rat over that which came directly from the chamber represented the water from the respiratory tract, and the remainder of the water absorbed from both systems, less that in the intake air (as determined in the blank), represented the water evaporated from the skin.

At the beginning of each experiment the air streams were passed through the chamber containing the rat during a preliminary equilibration period of twenty minutes, and after this time the drying tubes were put in place and the initial gas volumes were read. The experimental period lasted for thirty minutes, and at the end of this time the final volume readings were taken and the drying tubes were disconnected and weighed.

The results of the measurements, on three groups of five male albino rats weighing between 278 and 394 grams, are reported in table 1. With the animals of group I, anesthetized during the experiments with nembutal, 40 mgm. per kgm., intraperitoneally, or urethane, 1.5 grams per kgm. subcutaneously, the temperature of the air as it entered the chamber was 22.4 to 23.9°C. and the relative humidity was 47 to 58 per cent. With group II, which was given nembutal, 50 mgm. per kgm., the air temperature was 33.2 to 34.2°C. and the relative humidity was 48 to 55 per cent. The increased dose of nembutal given to this group was necessary because the animals came out of the anesthesia more rapidly at the higher environmental temperature. The temperature of the intake air with group III, which was given no anesthetic, was 23.3 to 25.3°C. and the relative humidity was 46 to 50 per cent.

The body temperatures of the animals in group I, taken with a rectal thermometer, dropped during the course of the experiments, but those of group II remained normal. The temperature ranges at the end of the experiments were 33.3 to 35.7°C. for the first group and 37.9 to 38.1°C. for the second.

DISCUSSION. In these experiments the average loss of water through the skin was 74 per cent of the total insensible water loss in each of the two groups of anesthetized rats and 54 per cent in the group which was not anesthetized. These results are similar to those found in man by Benedict and Benedict (5), Jores (6), Adachi and Ito (7) and Burch and Winsor (8).

The sweat glands of the rat, according to Hieronymi (9), are rudimentary and are not found in all parts of the skin; in this laboratory a limited number of skin sections have been studied by Dr. Henry Siegel, but no sweat glands were found. It may be concluded from this that the water loss through the skin of the rat is a process of diffusion rather than of secretion. The same conclusion was reached with regard to man by Loewy and Wechselsmann (10) who found, as did Richardson (11), that human subjects with no sweat glands lost water through the skin at a rate equal to that in normal subjects under the same conditions as long as the temperature was not high enough to cause active sweating.

In the experiments reported here the anesthetized rats lost a greater proportion of water through the skin than did the non-anesthetized rats, due probably to a decrease in respiratory volume with anesthesia and a greater relaxation, resulting in more complete exposure of the body surface to the surrounding air. While

the proportion of water lost through the skin was the same in the two anesthetized groups, the total insensible loss was much lower in group I. With these animals the combined effects of the anesthetic and the cooler environment resulted in a decreased metabolic rate, shown by their lowered body temperature. From the findings of Greene and Luce (1) and Greene (2), this would be expected to produce a smaller total insensible loss. The animals in group II were also anesthetized, but they were in a warmer environment, their body temperature did not drop, and their total water loss was the same as that of the non-anesthetized rats in group III.

The factors which influence insensible water loss have been reviewed by Newburgh and Johnston (12), and the loss under various conditions in small animals has been studied by Zak and Leiner (13). The loss from the respiratory tract is dependent upon the temperature and humidity of the inspired air and upon the respiratory volume, while the loss from the skin is influenced among others by the temperature, humidity, and rate of air flow, the peripheral blood flow, the water content of the body, and the character of the body covering. The factors which are under the control of the rat, however, have more weight than the environmental conditions (14). Although an evaluation of the relative amounts of water lost insensibly through the skin and from the respiratory tract applies only to the particular experimental conditions used, the results reported here show that the loss of water through the skin of the rat amounts to a large proportion of the total insensible loss.

SUMMARY

Separate determinations of the amounts of water lost insensibly through the skin and from the respiratory tract of the rat have been made under normal environmental conditions and under conditions approximating those of the tropics. The amount of water lost through the skin was 74 per cent of the total insensible loss in anesthetized rats under both temperate and tropical conditions, and 57 per cent in non-anesthetized rats under temperate conditions. No experiments were run with non-anesthetized rats under tropical conditions. In anesthetized rats under temperate conditions the body temperature dropped and the total insensible loss was lower than that of anesthetized rats under tropical conditions in which the body temperature remained normal. The total loss in the latter group was the same as that of non-anesthetized rats under temperate conditions. Since the rat has few if any functional sweat glands, the loss of water through the skin is probably a process of diffusion rather than of secretion.

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VENOUS RETURN IN THE ABSENCE OF CARDIAC DRIVE

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In 1929 Riml demonstrated that when the pulmonary artery of a rabbit was clamped, half the animal's blood could be recovered from a cannula in the jugular vein. This venous return in the absence of cardiac drive was not explained. He also observed a marked rise in venous pressure after clamping the pulmonary artery. He reported that after removing the clamp and returning the shed blood to the vascular system it was possible to repeat the experiment several times in one animal. Successive trials gave decreasing values either for yield of blood or for venous pressure.

In 1936 Henderson *et al.* reported similar experiments in four deeply narcotized cats (Nembutal, 40 mgm. per kgm.) in which there was "retention of nearly normal tonus as indicated by very active knee jerks." Using a trocar thrust into the right auricle, these workers reported that the first gush of blood emerged under a pressure sufficient to support a column of blood 10 or 12 cm. in height, and that this pressure was sufficient to drive more than half the blood volume to the right heart. They believed the experiments showed that skeletal muscle tone is largely responsible for the venous flow since a few minutes after death the phenomenon could not be observed. They speculated on the possible rôle of capillary elasticity in promoting venous return. Furthermore, they claimed that the vasomotor system was not involved since the result on a sympathectomized cat given to them by Dr. W. B. Cannon was the same as that in the three normal animals.

In connection with the experiments to be reported here, it was thought to be of interest to test further the importance of muscle tone by using curare, and by varying the depth of anesthesia, along with some of the procedures described by the above investigators. In addition, the rôle of the spleen in this type of experiment is briefly considered.

EXPERIMENTAL PROCEDURE. The present report is based on 37 observations in which modifications of the Riml procedure were used on 3 cats and 30 dogs. In most of the experiments the pulmonary artery and aorta were clamped or tied simultaneously. In the 3 cats and in 1 dog the blood was recovered through a 2.5 mm. trocar thrust into the right ventricle or by pipetting it from the chest cavity after it had escaped through a wide gash in the right ventricle. In the remaining 33 observations on dogs, blood was collected through a cannula in the azygos vein at the point where it joins the superior vena cava, the open end of the cannula being in the vena cava. After clamping the arteries, the blood from this cannula was collected into a reservoir held at about heart level. This reservoir contained heparin solution, or the dog was heparinized, and the blood

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was allowed to flow until it appeared to have stopped and the right heart looked empty. The blood was measured and in some cases returned immediately to the heart by gravity after removal of the clamp on the arteries.

Nine animals (1 cat and 8 dogs) received a large dose (5-9 mgm. per kgm.) of Intocostin (curare) to abolish skeletal muscle tone. The drug was demonstrated to be effective by the failure of muscular response to electrical stimulation of the sciatic nerve, by abolition of the knee jerk and by cessation of movements of the diaphragm.

In some experiments, the pedicle of the spleen was clamped to prevent expulsion of blood. Since minor manipulation of the spleen is liable to produce partial contraction, great care was taken to minimize handling. The spleen was generally allowed some time to relax after the clamps were in place. The clamps were not closed until inspection showed that the spleen was not contracted.

Table 1 summarizes the experiments showing the weights of the animals, the total volume of blood recovered, and this volume expressed as milliliters per kilogram of body weight.

The volume of blood expelled. Four animals (group I) were bled from the azygos vein with no tying or clamping of the arteries. From these animals blood was recovered to the extent of 44, 54, 43, and 41 ml. per kgm. of body weight, respectively. Four dogs (group II) whose hearts were caused to fibrillate by faradic excitation 23 minutes before clamping the vessels and bleeding, yielded 16, 21, 26 and 29 ml. per kgm., respectively. This is significantly less than the yield from the previous group, and indicates that about 23 ml. per kgm. less blood is propelled toward the heart in the animals in which the heart has stopped and death has occurred. Propulsive mechanisms other than the heart are the subject of this report. In our experiments the blood recovery was never as great as half the total estimated blood volume of the dog ($\frac{1}{2}$ body weight).

The skeletal muscles. In order to investigate the rôle played by skeletal muscular activity in the venous return, we have studied animals lightly anesthetized with cyclopropane² (groups VI and VII), animals more heavily narcotized with Nembutal (group III), and curarized animals (groups IV and V). In one instance (cat 3) the absence of skeletal muscle tone was still further ensured by the combination of curarization and pithing. The animals with diminished muscle tone as a result of treatment with Intocostin (groups IV and V), regardless of depth of anesthesia, yield less blood than those animals treated with light cyclopropane anesthesia alone (groups VI and VII). The exceptionally low yield of blood from dog 30 is not explained.

The influence of the spleen. In five tests on four animals (group VIII) under Nembutal anesthesia, with the spleen pedicle clamped, the amount of blood recovered was essentially the same as that obtained from Nembutalized animals without spleen pedicle clamping.

² Acknowledgment is made to the department of anesthesiology for the generous assistance and suggestions of Drs. K. C. O'Neal, M. V. Carroll and M. Yao.

TABLE 1

ANIMAL NO.	WT.	BLOOD RECOVERED	BLOOD RECOVERED	REMARKS
Group I Blood collected without occlusion of pulmonary artery and aorta				
	gm.	ml.	ml./kg.	
Dog 14	11.2	488	44	Nembutal anesthesia
Dog 22	13.1	706	54	Nembutal anesthesia
Dog 24	8.8	380	43	Nembutal anesthesia
Dog 25	7.5	310	41	Nembutal anesthesia. 2 cc. heparin i.v.
Average.....			46	
Group II Blood collected 23 minutes after heart was fibrillated				
Dog 15	11.0	175	16	2 cc. heparin i.v.
Dog 16	9.1	190	21	2 cc. heparin i.v.
Dog 17	11.4	300	26	2 cc. heparin i.v.
Dog 18	11.2	325	29	2 cc. heparin i.v.
Average.....			23	
Group III Nembutal anesthesia (Dose 30 mgm./kgm.)				
Dog 1	8.0	250	31	Blood from trocar in right ventricle
Dog 8	10.5	370	35	Trial I
Dog 10	7.2	205	28	Trial I
Dog 11	10.8	360	33	Trial I. Given oxygen
*Cat 4	2.2	50	*23	Ether. Trocar in right ventricle
*Cat 6	2.0	47	*24	Trocar in right ventricle
Average.....			32	
Group IV Nembutal anesthesia; Intocostin				
*Cat 3	3.0	61	*20	Ether. Brain and cord pithed
Dog 5	7.1	190	27	
Dog 8	10.5	275	26	Trial II
		325	31	Trial III
Dog 9	6.7	202	30	Trial I
		175	26	Trial II
		170	25	Trial III
Dog 20	11.5	260	23	Spleen pedicle clamped
Average.....			27	
Group V Light cyclopropane anesthesia; Intocostin				
Dog 30	11.7	155	13	2 cc. heparin i.v.
Dog 31	12.2	385	32	2 cc. heparin i.v.
Dog 33	12.1	410	34	2 cc. heparin i.v.
Dog 34	10.1	305	30	2 cc. heparin i.v.
Average.....			27	

TABLE 1—*Concluded*

ANIMAL NO.	WT.	BLOOD RECOVERED	BLOOD RECOVERED	REMARKS
Group VI Light cyclopropane anesthesia				
	kg.	ml.	ml./kg.	
Dog 19	13.0	510	39	Struggling at start of blood collection
Dog 23	10.0	360	36	
Dog 26	16.8	810	48	Head and neck movements. 2 cc. heparin i.v.
Dog 27	11.6	426	37	Head and neck movements. 2 cc. heparin i.v.
Average.....			40	
Group VII Light cyclopropane anesthesia; spleen pedicle clamped				
Dog 28	16.8	625	37	2 cc. heparin i.v.
Dog 29	4.3	170	40	2 cc. heparin i.v.
Average.....			39	
Group VIII Nembutal anesthesia; spleen pedicle clamped or tied				
Dog 10	7.2	208	29	Trial II
Dog 11	10.8	283	26	Trial II
Dog 12	12.6	305	24	Received some cyclopropane
Dog 13	7.4	255	35	Trial I. Received some cyclopropane
		250	34	Trial II
Average.....			30	

* Not included in average figures.

In two animals (group VII) the pedicle of the relaxed spleen was clamped but light cyclopropane anesthesia was employed, and the animals showed muscular movements of the legs, trunk and neck. The movements occurred before or immediately after occlusion of the pulmonary artery and aorta and were not attributed to cerebral anemia. The volume of blood expelled was the same as that obtained from other lightly anesthetized animals whose spleens were undisturbed but was greater than that from Nembutalized animals whether the spleen was clamped or not.

Pressure. Riml and Henderson were both impressed in their experiments by the pressure under which the blood gushed out of the right heart. More striking in our experiments was the fact that a difference of level of a centimeter or so between the blood in the collecting reservoir and the caval orifice makes a very great difference in the rate of collection of blood and perhaps also in the total yield.

DISCUSSION. In this series of modified Riml experiments, the variability of the yields makes it difficult to quantitate the differences between observations made on different animals, and the procedures used are so drastic that one hesitates to attach too much numerical significance to small differences or to successive trials on one animal.

It is evident that the contribution of the spleen to the extra blood recovered from the struggling animal as contrasted with the dead animal is so small that it probably is not significant. Four Nembutalized dogs with intact splenic circulation yielded an average of 32 ml. blood per kgm., whereas four dogs with occluded splenic circulation yielded 30 ml. per kgm. In the group of lightly anesthetized animals the difference in average blood yields, with and without splenic vessel ligation, was only 1.0 ml. per kgm.

A very large proportion of the blood which can be drained from the azygos vein in the living animal with intact circulation can still be obtained after cardiac drive is eliminated by clamping the pulmonary artery and aorta. Animals under light anesthesia in which there was noticeable skeletal muscle activity yielded the greatest average amount of blood after eliminating cardiac drive. Depression of skeletal muscle activity by deeper anesthesia or by the use of curare, or even elimination of all skeletal muscle tonus by death, did not prevent the recovery of an amount of blood equal to about half that which is recovered from animals with no arterial ligation. Therefore, the force propelling this half of the total volume of blood, which issues even when the arteries are clamped, must be independent of the activity of the skeletal muscles.

Perhaps this force arises in the capillaries and other tissues whose elastic properties may persist after death.

SUMMARY

Blood was drained from the azygos vein of dogs in a modified type of Riml¹ experiment. The amounts of blood recovered were compared in groups of animals subjected to the following procedures: 1, pulmonary artery and aorta clamped under light anesthesia, 40 ml. per kgm.; 2, arteries clamped under deep anesthesia, 32 ml. per kgm.; 3, arteries clamped in curarized animals, 27 ml. per kgm.; 4, arteries and spleen pedicle clamped, under deep anesthesia, 30 ml. per kgm.; 5, arteries and spleen pedicle clamped under light anesthesia, 39 ml. per kgm.; 6, dead animals, arteries clamped 23 minutes after experimentally induced ventricular fibrillation, 23 ml. per kgm. A control group was bled without any arterial clamping and yielded 46 ml. per kgm.

Splenic contraction contributes very little, if any, blood to the total yield, but skeletal muscle activity accounts, apparently, for about half the total yield.

Elastic forces persistent in tissues even after death, may account for the remainder of the blood yield.

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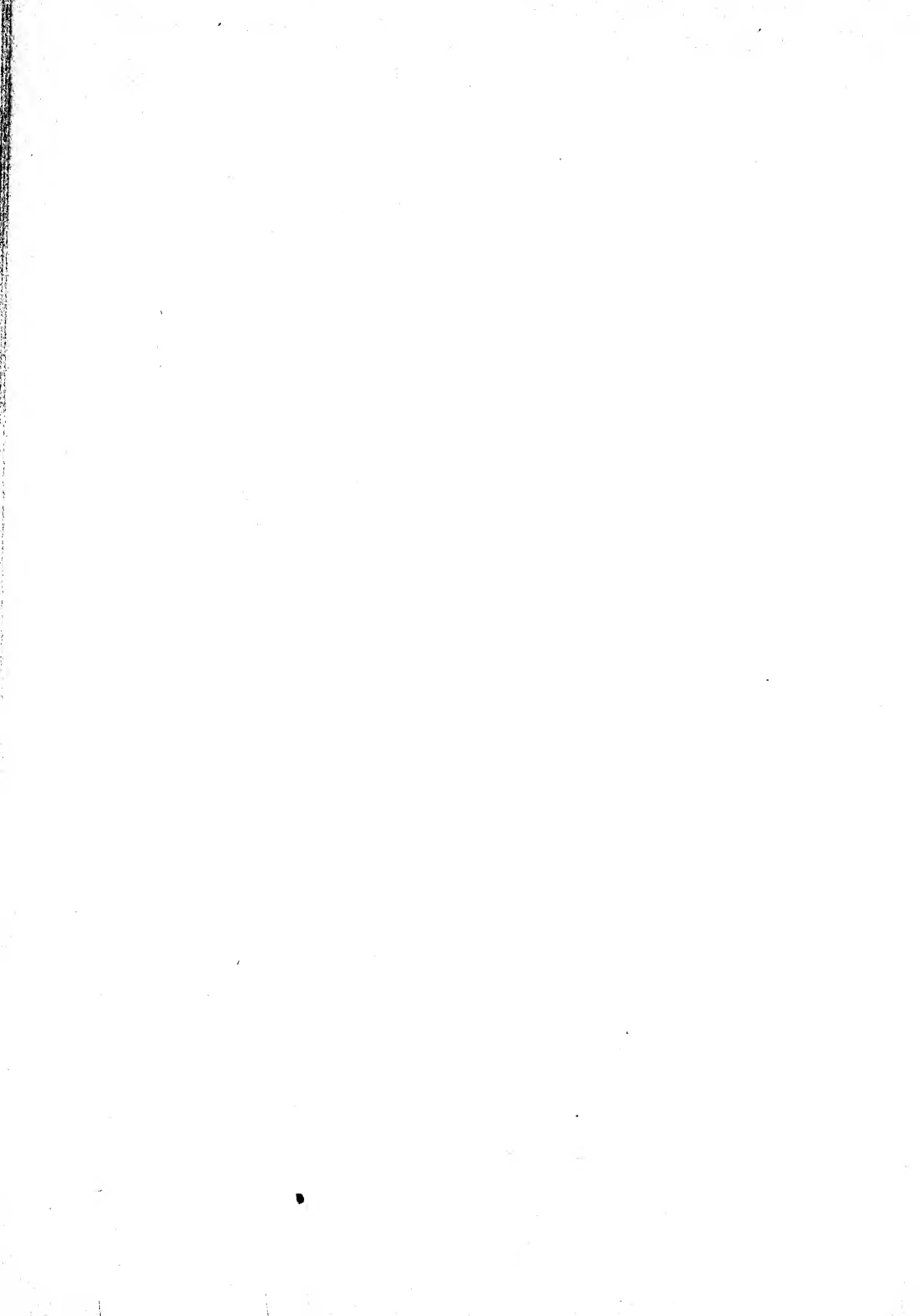
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ERRATUM

Volume 145, page 239: The Fate of CO in the Body During Recovery from Mild Carbon Monoxide Poisoning in Man, by F. J. W. Roughton and W. S. Root.

The authors desire to amend the sentence beginning on line 24 of page 249 to read:

"Furthermore, according to recent theoretical work by one of us (16) it is probable that the rate of distribution of CO from the red cells to the myoglobin is faster than some writers have supposed, for the calculated half-time of reaction is of the order of 200 seconds, which is short compared with the tempo of the slow progressive loss of CO from the blood, since this has a half-time of about 240 minute when breathing air."



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THE EFFECT OF ELECTRICAL STIMULATION UPON THE COURSE OF ATROPHY AND RECOVERY OF THE GASTROCNEMIUS OF THE RAT¹

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It has now been well established by the experimental work of the last several years that the loss of weight and strength of experimentally denervated muscle can be effectively retarded by the use of electrical stimulation under appropriate conditions (1-7). These conditions are: 1, a current must be used which will produce maximal tension in the muscle within the limits of tolerance of the animal; 2, the muscle must be stimulated at frequent periods, 3, treatment must be started soon after denervation. The contradictory reports of other investigators (8, 9, 10) are probably due to a failure to observe one or more of these precautions.

Since the principal use of electrical stimulation of muscle as a therapeutic measure is indicated in those instances where the loss of motor function is temporary, it is of practical importance to determine whether such treatments exert any beneficial influence upon the return of motor function when neurotization occurs. It is difficult properly to evaluate the extent of return of voluntary movement at various stages of recovery in the experimental animal. Using the criteria of muscle weight and fibre size, Gutmann and Gutmann (7) have shown that demonstrable differences were still present between the treated and untreated muscles of rabbits after primary suture of their peroneal nerves, four months after direct current (galvanic) exercise had been stopped. Hines and others (8) have recently reported that daily faradic stimulation of the rat gastrocnemius following crushing of the tibial nerve effectively retarded loss of weight and strength although the difference between the treated and untreated muscles was equalized thirty-five days following the lesion.

The present experiments were designed to study the effect of electrical stimulation upon the course of recovery of the denervated gastrocnemius of the rat using weight and strength as an index when the nerve was allowed to regenerate.

METHODS. Male albino rats weighing from 225 to 275 grams were used. The sciatic nerve was crushed bilaterally with mosquito forceps at the sciatic notch.

¹ Aided in part by a grant from the National Foundation for Infantile Paralysis.

The forceps were kept in place for five minutes. In every case, the location and conditions of crushing were kept as identical as possible.

In a preliminary study to determine the variability of weight and strength between left and right gastrocnemii during the course of degeneration and recovery, one group of rats received no treatment following the nerve crush. They were sacrificed fourteen, twenty-one, twenty-eight, thirty-five and forty-two days postoperatively and muscle tension and wet weight were determined for both gastrocnemii. Tension was measured by an isometric myograph of the torsion type with the muscle subjected to maximal stimuli of 25 or 50 cycle A.C. Since maximal tension of denervated muscle in the rat is obtained with a 25 cycle A.C. and maximal tension of normal muscle with a 50 cycle A.C., both frequencies were used in testing the strength of the recovering muscle.

In those animals receiving treatment, the muscle on one side was stimulated for five minutes twice daily with a modulated 25 cycle A.C. at 1.5-2.0 m.a., which produced forty contractions a minute. Treatment was started the day following the operation and was continued until the day preceding sacrifice, except in the case of the thirty-five and forty-two day rats where treatment was discontinued on the 28th day.

In order to carry out the stimulation without the use of an anesthetic, the rats were placed in plaster shells which were then wrapped with elastic bandage, leaving the leg to be stimulated unsecured and easily movable. After several treatments the rats showed little resistance against this method of restraint and would lie quietly without struggle during the course of the treatment. The stimulating electrode consisted of an insulated copper wire covered at one point by saline soaked cotton. The wire was fitted around the ankle of the rat and when fastened in a clamp served to hold the leg in position. A large dispersive electrode was placed on the abdomen. The foot was loaded with a 25 gram weight.

The rats were sacrificed fourteen, twenty-one, twenty-eight, thirty-five and forty-two days postoperatively and the wet weights and tensions of both muscles determined in the manner described above.

In one group of ten animals, treatments were not started until twenty-one days following crushing of the nerve. The treatments were continued until the 35th day when the animals were sacrificed.

RESULTS. I. *Comparison of weight and tension of untreated right and left gastrocnemii following the crushing of the sciatic nerve.* The results of this group of experiments are summarized in table 1. The mean differences in weight and tension between right and left muscles at various stages of atrophy and recovery are not significantly different from the values found for the normal muscles. The method used to produce the denervation did not lead to such wide differences during degeneration and recovery as would obscure any effects of the electrical stimulation upon the weight and strength of the treated muscles. The mean differences and their standard errors have been used to evaluate the significance of differences between treated and untreated muscles.

The rate at which the weight and tension of the gastrocnemius are lost in the

first fourteen days following the nerve crush are similar to those reported previously by us (9) following complete section of the sciatic nerve. Loss of tension occurs more rapidly and to a greater extent than the loss of weight. Conversely, as recovery occurs, the increase in tension occurs at a greater rate than the increase in weight.

II. *Effect of electrical stimulation upon weight and strength loss.* The results of this group of experiments are summarized in table 2 and figure 1. Forty-two days following the nerve crush and two weeks after the last treatment, the

TABLE 1

NO. OF RATS	DAYS AFTER NERVE CRUSH	% DIFFERENCE OF WEIGHT BETWEEN RIGHT AND LEFT MUSCLES	% OF NORMAL* WT. (AVE. OF RT. AND LEFT MUSCLES)	% DIFFERENCE TENSION BETWEEN RIGHT AND LEFT MUSCLES	% OF NORMAL† TENSION (AVE. OF RT. AND LEFT MUSCLES)
25	Normal	2.1 \pm 0.35	100.0	6.7 \pm 0.94	100.0
15	14	3.9 \pm 0.67	55.3	8.1 \pm 0.18	30.1
10	21	5.3 \pm 0.90	49.1	8.7 \pm 1.5	28.7
10	28	5.6 \pm 0.80	54.6	8.4 \pm 2.1	34.9
15	35	5.4 \pm 0.73	66.2	7.3 \pm 1.16	53.6
12	42	5.9 \pm 0.85	72.8	7.7 \pm 1.6	57.8

* Determined on basis of $\frac{\text{Normal muscle weight (gms.)}}{\text{Body weight (gms.)}} = 0.58\%$.

† Determined on basis of $\frac{\text{Normal muscle tension (gms.)}}{\text{Body weight (gms.)}} = 8.7 \pm 0.26$.

TABLE 2

NO. OF RATS	DAYS AFTER NERVE CRUSH	% DIFFERENCE BETWEEN TREATED AND CONTROL MUSCLES		% OF NORMAL WEIGHT		% OF NORMAL TENSION		% TENSION (INDIRECT STIM.)	
		Weight	Tension	Control	Treated	Control	Treated	Control	Treated
15	14	25.8	47.8	57.4	71.7	32.0	46.6		
15	21	41.6	53.4	50.2	71.8	30.4	47.3	57.1	55.5
15	28	35.8	51.5	53.5	72.7	36.2	52.5	58.9	60.7
20	35	20.3	22.4	64.8	77.2	55.8	68.0	79.9	81.4
20	42	16.2	20.6	70.2	81.3	59.7	72.6	83.7	83.5
10*	35	4.4	7.6	65.9	66.7	53.6	55.4	80.4	79.1

* Treatments started 21 days after nerve crush and continued until 35th day.

stimulated muscles still maintained a significant though narrowing advantage in weight and strength over the untreated control. Between the 21st and 42nd days during which time the rate of recovery had markedly accelerated, the weight and tension of the control muscles increased 29.3 per cent and 65.0 per cent respectively; that of the treated muscles, 11.2 per cent and 39.2 per cent. Thus although the rate of recovery of the untreated muscle is apparently greater during this period, the treated muscles have the distinct advantage of recovering at higher levels of weight and tension. From the thirty-fifth day the rates of

recovery for both muscles are the same and should this continue it might be expected that the treated muscles would reach normal values at an earlier period.

Although the differences in tension between the treated and untreated muscles are much greater than the differences in weight, muscle weight is more effectively maintained at levels approaching normal values than is tension.

If the ratio of the tension developed by direct and indirect stimulation of the muscle (last column, table 2) is an indication of the extent of neurotization, then there is no evidence that the degree and rate of re-innervation are at all influenced by electrical stimulation.

The group of rats in which treatment was started 21 days postoperatively and continued until the thirty-fifth day, showed no significant differences in weight and tension between the treated and untreated muscles.

DISCUSSION. The results of these experiments clearly indicate that electrical stimulation of denervated muscle, when properly conducted, results in a stronger

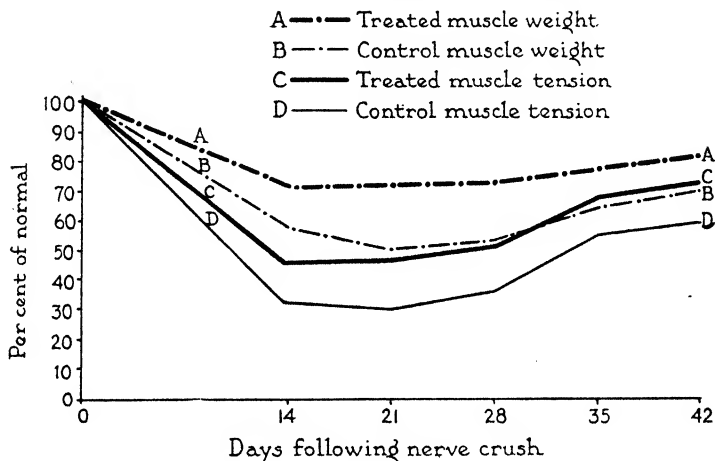


Fig. 1

and heavier muscle not only during the period of complete denervation but also well into the period of regeneration of the peripheral nerve. Since there is no experimental evidence that electrical stimulation influences in any way the regeneration processes of the motor nerve, the rationale for the use of electrical stimulation in the treatment of paralyzed muscle must be based upon the facilitation of re-education and voluntary use when neuromuscular connections have again been established. Whether the maintenance of the weight and strength of denervated muscle does promote an earlier functional recovery cannot be established conclusively either by the experiments reported here or by the observations of other investigators. It would appear, however, to be a reasonable assumption that a muscle in which atrophy has been prevented to a considerable extent would lend itself more readily to the process of re-education and to the return of normal voluntary use. The proof of such an assumption must depend eventually upon similar experiments carried out on animals in which

functional recovery can be accurately evaluated and upon carefully controlled clinical observations in man.

It should be emphasized that the differences in weight and tension obtained in these experiments occurred under conditions which were most favorable for the earliest regeneration of the peripheral nerve. Such uncomplicated interruptions of the nerve supply to a muscle are not usually encountered clinically. More commonly, complicating factors of hemorrhage, trauma, edema, etc., may delay the regenerative processes for considerable periods. In the experiments of Gutmann and Gutmann (7), cited previously, the differences in weight and fibre size between the treated and untreated muscles became more marked when the union of the nerve was delayed by a secondary suture.

When electrical stimulation is delayed until re-innervation of the muscle has already started, it apparently is without appreciable effect upon the subsequent recovery of weight and tension of the muscle. It seems probable that electrical stimulation does not reinforce the natural recovery of those muscle fibres which have already been neurotized; nor is it effective in increasing the weight and strength of the remaining non-innervated fibres which have undergone considerable atrophy. It would be erroneous to conclude from this that electrical stimulation which was instituted soon after denervation should be discontinued when the first signs of neurotization appear. Electrical stimulation should still prove effective in maintaining those muscle fibres as yet without innervation.

SUMMARY

1. When the sciatic nerve is crushed bilaterally under identical conditions the loss of weight and tension for both right and left gastrocnemii are approximately equal.

2. Loss of tension occurs at a more rapid rate and to a greater extent than weight loss. Conversely, tension returns more rapidly as the nerve regenerates.

3. Appropriate electrical stimulation of the gastrocnemius muscle of the rat results in a heavier and stronger muscle both during the period of denervation and recovery.

4. Appropriate electrical stimulation when started after re-innervation has occurred is without effect upon the subsequent course of recovery.

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THE INFLUENCE OF ADRENALIN ON PLASMA PROTHROMBIN

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In a study of factors affecting the coagulation time of the blood, Cannon and associates (1) reported over thirty years ago that adrenalin in small doses, intravenously, and larger doses, subcutaneously, would hasten the coagulation of blood. However, they noted that when the circulation was confined to parts above the diaphragm, or if the liver and intestines were removed, the hastening of coagulation could no longer be produced by adrenalin. They explained that adrenalin liberated some factor or factors from the liver which accelerated the process of coagulation. They (2) further reported that stimulation of the splanchnic nerves resulted in shortening of the coagulation time of the blood, and that repeated stimulation produced less marked effects. In the absence of the adrenal glands, splanchnic stimulation did not shorten the coagulation time, nor did increase of adrenalin, in the absence of the liver and intestines, shorten the coagulation time. Therefore, they concluded that splanchnic stimulation shortened the coagulation time of the blood by the action of the discharged adrenalin from the adrenal glands on the liver (and intestines?).

In a communication on nervous regulation of the clotting mechanism, de Takats (3) stated that fear, apprehension, nervous strain, and hemorrhage increased the tendency toward thrombosis. He reported that intravenous administration of epinephrine markedly inhibited the reaction to heparin in two hypertensive patients. He suggested that since plasma prothrombin was manufactured by the liver, sympathetic nervous stimuli might possibly discharge prothrombin from the liver. He further stated that this view was supported by the observation of an increase in the level of prothrombin after epinephrine or neo-synephrine. However, he considered other possible mechanisms that could have been involved.

Link (4) stated that adrenalin could hasten the restoration of prothrombin time when given to animals about to bleed from excessive doses of dicumarol. He expressed the opinion that administration of adrenalin when hemorrhage from dicumarol was threatened might be clinically useful.

Moses (5) reported that the administration of 1 mgm. of epinephrine intravenously caused no significant or consistent change in the heparin tolerance curves of five individuals receiving 0.15 mgm. of heparin per kgm. of body weight.

The purpose of this study was to find out what effect the administration of adrenalin would have on the prothrombin content of normal blood of control animals, and of these same animals after they were under the influence of intravenously administered dicumarol.

METHODS. Dogs weighing between 6 and 12 kgm. and maintained on the ordinary kennel diet, and rats weighing about 200 grams each, were used in this

study. The Quick method as modified by Pohle and Stewart (6) was employed for the determination of the prothrombin time of diluted (12.5 per cent) plasma both before and after intravenous administration of adrenalin. A volume of 1.8 cc. of blood was withdrawn from each dog into a syringe containing 0.2 cc. of 0.1 molar sodium oxalate solution, and then centrifuged. Control plasma prothrombin times were taken on all dogs, and then each was given an intravenous injection of adrenalin chloride solution. The dosage and time after injection appear in the tables of results. After control values were established, before and after administration of adrenalin, each of the dogs was given intravenously a single dose of an alkaline 0.5 per cent solution of the disodium salt of dicumarol

TABLE 1

Control and daily values of dilute (12.5%) plasma prothrombin clotting time in seconds before and during the 2nd or 3rd minute after daily intravenous injection of 0.05 mgm./kgm. adrenalin into dogs before and after the administration of a single dose of dicumarol intravenously

DOG NUMBER	Controls		DILUTE PLASMA (12.5%) PROTHROMBIN TIME IN SECONDS																							
			B*	A*	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A
	B	A																								
1	25	25	72	69	144	110	146	147	180	204	164	144	52	52	40	43	34	34	30	27	27	35	25	25	25	25
2	26	28	71	71	70	160	142	215	187	225	217	203	205	60	56	43	46	34	34	29	28	27	28	26	28	28
4	23	26	84	84	168	141	239	241	277	304	318	267	257	234	97	8	25	30	27	26	21	23	24	25	25	25
5	25	25	48	50	130	116	152	135	186	171	110	115	50	53	32	36	32	34	32	30	32	31	28	28	28	28
7	26	28	52	50	140	122	177	159	195	190	83	93	46	43	37	32	34	32	34	35	32	34	27	28	28	28
8	23	26	56	56	135	140	215	186	191	199	89	94	79	71	32	38	32	34	32	31	29	27	30	26	26	26
Average..	24	26	64	63	146	128	191	176	209	214	161	153	91	85	47	50	32	33	31	29	28	30	27	27	27	27
Days after dicumarol injection.....				1	2	3	4	5	6	7	8	9	10	11												

* B = Blood sample taken before injection of adrenalin.

A = Blood sample taken after injection of adrenalin.

Sample A was taken during the 2nd minute in dogs 1, 2, and 4, and during the 3rd minute in dogs 5, 7, and 8 after adrenalin injection.

amounting to 15 mgm. of dicumarol per kgm. of body weight. Thereafter, plasma prothrombin times were determined daily before and after administration of adrenalin, until the prothrombin times returned to the initial levels prior to the injection of dicumarol.

For the prothrombin time in rats, a volume of 0.9 cc. of blood was withdrawn from the heart into a syringe containing 0.1 cc. of 0.1 molar sodium oxalate solution, before and after injection of adrenalin into the tail vein, intramuscularly, or subcutaneously. The dose of adrenalin injected, the route of administration, and the time the blood sample was withdrawn from the rats after administration of adrenalin are specified in the tables of results.

RESULTS. Table 1 presents individual and average prothrombin times on six

TABLE 2

Control and daily values of dilute (12.5%) plasma prothrombin clotting time in seconds before and 5, 10, 15 and 20 minutes after the administration of adrenalin intravenously into dogs before and after the administration of a single dose of dicumarol intravenously

ADRENALINE mgm./kgm.	DOG NUMBER	DILUTE PLASMA (12.5%) PROTHROMBIN TIME IN SECONDS															
		Controls		Dicumarol 15 mgm./kgm. I.V.													
		B*	A5*	A10*	B	A5	A10	B	A5	A10	B	A5	A10	B	A5	A10	B
0.001	9	22	19	20	103	78	155	150	126	262	151	227	164	127	152	135	105
0.001	10	24	20	20	94	83	185	112	160	278	221	104	196	226	177	70	85
Average....		22	19	20	98	80	170	131	143	270	186	210	180	176	164	102	95
0.001	11	B	A15*	A20*	B	A15	A20	B	A15	A20	B	A15	A20	B	A15	A20	B
0.001	12	24	21	21	92	77	132	138	150	211	185	207	56	57	66	37	38
Average....		25	24	24	90	86	143	135	154	187	187	209	94	100	119	35	36
0.030	13	B	A5	A10	B	A5	A10	B	A5	A10	B	A5	A10	B	A5	A10	B
0.030	14	27	22	24	103	92	189	190	190	232	212	245	143	131	127	34	34
Average....		27	24	24	115	88	215	180	182	219	172	218	174	179	160	79	64
0.030	15	B	A15	A20	B	A15	A20	B	A15	A20	B	A15	A20	B	A15	A20	B
0.030	16	23	20	21	110	128	197	155	170	195	275	188	46	50	49	29	33
Average....		22	21	20	126	94	205	150	133	253	130	213	141	226	152	80	95
		22	21	20	118	111	201	152	151	224	202	150	93	138	100	45	64
Days after dicumarol injection		1		2		3		4		5		6		7		8	

* B = Blood sample taken before injection of adrenalin.

A5, A10, A15, and A20 = Blood sample taken 5, 10, 15, and 20 minutes after injection of adrenalin.

dogs before and after intravenous injection of adrenalin (0.05 mgm./kgm.); also before and after administration of dicumarol. During the second or third minute after injection of this dose of adrenalin there was no significant change

TABLE 3

Differences of average plasma prothrombin clotting time in seconds between blood samples taken daily before, and at intervals after administration of adrenalin to dogs

DAYS AFTER DICUMAROL	TIME BLOOD WAS WITHDRAWN AFTER INJECTION OF ADRENALIN						ADRENALIN DOSE mgm./kgm.
	2 min.	3 min.	5 min.	10 min.	15 min.	20 min.	
Controls	+1	+1					0.050
			-3	-3	-1	-2	0.030
			-3	-2	-1	-1	0.001
1st	-2	0					0.050
			-1	-3	+6	+9	0.030
			+8	+7	-1	+3	0.001
2nd	-26	-9					0.050
			+3	-24	-22	-29	0.030
			-4	-22	-1	-5	0.001
3rd	-8	-7					0.050
			-35	-33	-49	50	0.030
			-39	-27	-8	+11	0.001
4th	+15	+3					0.050
			-47	-1	-22	-74	0.030
			-84	-60	0	+22	0.001
5th	-23	+2					0.050
			+5	-14	+45	+7	0.030
			-4	-16	+6	+25	0.001
6th	-9	-6					0.050
			-15	+4	+19	+18	0.030
			-7	+8	+1	-1	0.001
7th	+6	+1					0.050
			-2	0	-4	-1	0.030
			+2	+3	0	+2	0.001
8th	+2	0					0.050
			+1	0	-1	-1	0.030
			+1	+1	0	+1	0.001

in the plasma prothrombin time, either in the normal or after production of hypoprothrombinemia by dicumarol.

Table 2 shows that when adrenalin was given in doses of 0.001 or 0.030 mgm. per kgm., and dilute plasma prothrombin times were determined before, 5 and 10,

and 15 and 20 minutes after administration of adrenalin, the effects on prothrombin activity were variable and insignificant since they fall within the range of errors of the method.

Table 3 shows the variable differences in prothrombin time of dilute plasma taken before and after administration of adrenalin to dogs. It clearly indicates that when the prothrombin content of plasma is within the normal range or only slightly prolonged by dicumarol, the effect of adrenalin is insignificant.

Table 4 demonstrates the effects of different dosages of adrenalin administered intravenously, intramuscularly or subcutaneously into rats whose plasma prothrombin time was determined before and after injection of adrenalin. The

TABLE 4
Showing the effect of adrenalin on the prothrombin clotting time in dilute (12.5%) plasma in rats

RAT NUMBER	DILUTE PLASMA (12.5%) PROTHROMBIN TIME IN SECONDS			DOSE IN MG.M. OF ADRENALIN	MANNER OF ADMINISTRATION	TIME IN MIN. OF BLOOD SAMPLE AFTER ADRENALIN
	Before adrenalin	After adrenalin	Difference			
1	41	43	+2	0.05	Intravenous	3
2	38	39	+1	0.05	Intravenous	10
5	34	32	-2	0.0005	Intravenous	2
6	40	41	+1	0.0005	Intravenous	15
7	42	42	0	0.0005	Intravenous	20
10	36	35	-1	0.001	Intravenous	10
11	40	42	+2	0.001	Intravenous	15
14	39	39	0	0.000005	Intravenous	10
17	40	45	+5	0.00001	Intravenous	20
18	41	43	+2	0.00001	Intravenous	5
19	33	28	-5	0.00001	Intravenous	5
20	46	44	-2	0.0001	Intravenous	10
22	76	80	+4	0.0001	Intravenous	15
27	43	37	-6	0.1	Intramuscular	15
29	39	45	+6	0.1	Intramuscular	5
31	41	39	-2	0.01	Subcutaneous	10
33	43	39	-4	0.001	Subcutaneous	20
34	39	42	+3	0.001	Subcutaneous	15

differences between the prothrombin time before and after adrenalin are variable and fall within the range of errors of the method. Therefore, we feel justified in considering these differences insignificant.

SUMMARY AND CONCLUSIONS

The effects on plasma prothrombin of different doses of adrenalin intravenously administered into dogs have been studied in normalcy and after production of hypoprothrombinemia by dicumarol. In albino rats the effects on plasma prothrombin of different doses of adrenalin administered intravenously, intramuscularly, and subcutaneously have been studied without the production of hypoprothrombinemia by dicumarol.

The data obtained from this study justify the conclusion that in dogs and in albino rats the different doses of adrenalin employed produce no significant changes in prothrombin time of dilute plasma in normalcy; and that in dogs, made hypoprothrombinemic by dicumarol, the effects of adrenalin are variable and inconsistent and therefore probably insignificant.

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THE CARDIAC OUTPUT IN MAN: A STUDY OF SOME OF THE ERRORS IN THE METHOD OF RIGHT HEART CATHETERIZATION¹

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The extensive use of right heart catheterization has demonstrated that it is a safe and useful tool in the study of cardiovascular dynamics in human subjects (1, 2, 3). Klein (4) first utilized this method to obtain samples of mixed venous blood for the determination of the cardiac output. The direct Fick principle was applied and the cardiac output calculated as follows:

$$\text{cardiac output} = \frac{\text{oxygen consumption}}{\text{arterial O}_2 \text{ content} - \text{mixed venous O}_2 \text{ content}}$$

An analysis of the validity of this method must include 1, determination of the range of technical variation of each of the measurements employed; 2, evaluation of the right atrial blood as a true sample of mixed venous blood, and 3, a consideration of the actual variation in cardiac output from minute to minute in the resting subject. This paper is an appraisal of certain features of the catheter technique of cardiac output determination as based on our experience with the method in over 500 subjects.

METHODS. A detailed description of the method used in the cardiac output determination has been published elsewhere (3). A special ureteral type catheter with an angulated tip was passed up the venous system and into the right side of the heart. The position of the tip in the right atrium was verified by fluoroscopic examination. The passage of the catheter into the right ventricle was demonstrated by the change in position of the tip and by the higher pressure with distinct systolic pulsations. Since small amounts of saline solution were slowly passing through the catheter during the procedure, several cubic centimeters of blood had to be withdrawn through the catheter and discarded before the actual sample for analysis was obtained. This avoids contamination of the sample by the saline solution.

Arterial blood specimens were obtained from an inlying needle in the femoral artery, which had previously been well anesthetized with novocaine. Both venous and arterial specimens were collected under oil, iced, and analyzed for oxygen content by the method of Van Slyke (5) as soon as possible. All determinations were made in duplicate, usually by two technicians, and only those values checking within 0.1 volume per cent or less accepted as satisfactory.

¹ The work described in this paper was done under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Emory University School of Medicine.

Oxygen consumption was measured by collecting a two or three minute sample of expired air in a Douglas bag and by analyzing its contents by the method of Haldane (5).

In the studies reported here, the samples were obtained in rapid succession while the subject was in a relatively stable state. They were obtained from normal subjects and patients with a wide variety of disease conditions. The blood specimens were obtained from 1 to 20 minutes apart, usually 10 or less. For convenience in presentation, the initial sample was considered as the basis of comparison for subsequent samples from the same site or from another chamber of the heart in the same patient.

RESULTS. Thirty comparative studies on paired specimens of arterial blood were carried out. In none did the variation from the initial specimen exceed 0.4 volume per cent. Seventy-seven per cent of the determinations varied 0.2 volume per cent or less.

In 111 instances comparative determinations on samples of blood from the right atrium were made (fig. 1). Of these, 71 (64 per cent) showed a variation of 0.2 volume per cent or less. Eighty-six (78 per cent) varied no more than 0.4 volume per cent. The remainder (25 determinations or 22 per cent) varied as much as 2.3 volumes per cent.

Twenty-five comparative samples of ventricular blood were studied (fig. 1). Of these, 17 (68 per cent) varied no more than 0.2 volume per cent and 20 (80 per cent) no more than 0.4 volume per cent. The remaining 5 (20 per cent) varied as much as 1.8 volumes per cent.

The results of comparative studies of atrial and ventricular blood specimens in 19 patients are shown in table 1. In these subjects the catheter was first passed into the right ventricle. After samples of blood were obtained, the catheter was withdrawn to a position in the atrium and additional samples procured. The position in the atrium of the tip of the catheter was checked by fluoroscopy. In many instances continuous pressure tracings with the Hamilton manometer were recorded and the catheter withdrawn to a point where the tracings first assumed an atrial contour. The position of the tip of the catheter at that time corresponded to that described by Courmand *et al.* (1) as near the tricuspid valve.

In 11 patients the atrial and ventricular samples agreed within 0.4 volume per cent and in 17 they were within 1.0 volume per cent. In 2 patients, however, the oxygen content of the 2 specimens varied 1.2 volumes per cent. In the majority of instances the oxygen content of atrial blood was higher than that of the ventricular blood.

Forty-two duplicate samples of expired air were obtained and analyzed. Thirty-one of the duplicate determinations checked within 10 per cent. The remaining 11 varied as high as 20 per cent. There was no consistent tendency for the second determination to be either more or less than the initial one. The variation was not directly related to the volume of air ventilated, although in certain instances an increase in oxygen consumption was associated with increased respiratory volume.

DISCUSSION. It is not the purpose of this paper to discuss the validity of the Fick principle itself. It is well, however, to point out that under certain rare circumstances the value obtained is not necessarily that of the output of the left ventricle. When the output of the two ventricles is not equal, such as in atrial septal defect, the value obtained is actually that for the output of the right ventricle (6). In other situations, such as aortic valvular insufficiency, the value determined is the effective output; that is, the total output minus that

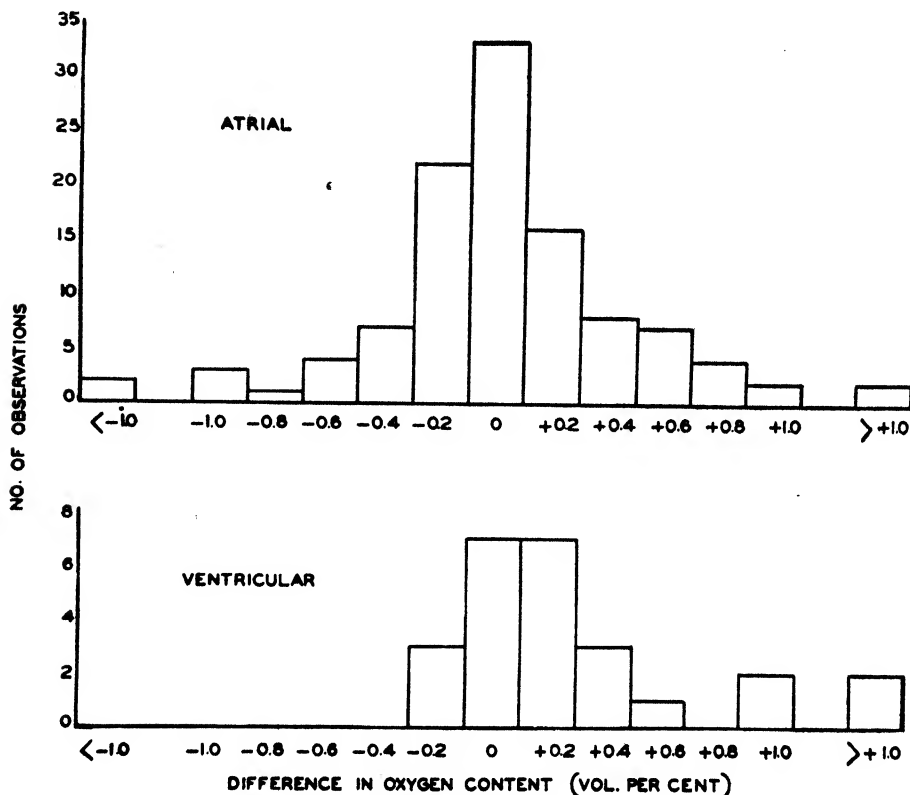


Fig. 1. Variations in oxygen content of serial samples of blood obtained from the right atrium and right ventricle.

which returned to the left ventricle through the insufficient aortic valve. Fortunately in most instances, including those of pulmonary disease with arterial oxygen unsaturation, the application of the principle is valid.

Although the Fick principle may be applied to determine the cardiac output from either oxygen or carbon dioxide data, we have used the former exclusively. This has been done because of the extreme lability of carbon dioxide levels in the body. In addition the technical difficulties in preservation of carbon dioxide

concentration in blood for analysis appear to be greater than with oxygen. We have had little difficulty in the determination of oxygen content of blood samples by the technique used in this study. The data recorded here are routine technical results obtained in the course of a clinical study of circulatory disorders, and do not represent special attempts for accuracy of the chemical methods employed. For routine practice we have continued to collect specimens under oil because of its technical simplicity, rather than the theoretically more desirable method of preserving them over mercury. The specimens were iced and analyzed as soon as possible.

TABLE 1

Difference in oxygen content of comparative samples of ventricular and atrial blood

NUMBER	BLOOD OXYGEN CONTENT			TIME INTERVAL
	Ventricular	Atrial	Difference	
	<i>volumes per cent</i>			<i>minutes</i>
1	13.6	13.9	0.3	1½
2	10.0	11.2	1.2	6
3	6.4	6.7	0.3	2½
4	10.2	10.9	0.7	4
5	10.5	10.5	0.0	7
6	5.6	6.6	1.0	3
7	6.8	7.2	0.4	1½
8	8.4	9.0	0.6	1
9	12.6	13.3	0.7	1½
10	7.7	6.5	-0.8	2
11	7.2	7.7	0.5	2
12	6.5	6.8	0.3	2
13	13.2	13.0	-0.2	9
14	12.9	12.0	-0.9	2
15	18.6	18.5	-0.1	3
16	7.0	7.4	0.4	9
17	9.8	10.7	0.9	9
18	6.0	6.4	0.4	4
19	14.3	14.0	-0.3	14

It is our opinion that the fluctuations in arterial oxygen content reported here are primarily technical, there being little actual variation in the oxygen content of arterial blood in subjects under these conditions. Using a continuous spectrophotometric method Drabkin and his colleagues (7) noted this stability in arterial oxygen content to be true in human subjects. Although no evidence of variation in the red blood cell content in our specimens could be demonstrated by the hematocrit readings, the possibility exists that minor changes in red blood cell content may have caused some of the variation in oxygen content of the arterial samples. For example, an increase in hematocrit reading from 42.0 to 42.5 would account for a change in oxygen content of about 0.2 volume per cent. Hematocrit variations of this magnitude or less are almost impossible to detect by the usual methods. An analysis of our data failed to reveal any evidence of

relationship of the deviations to time; in other words, the variation between samples was similar whether the interval between them was 1 or 10 minutes.

The samples of blood from the right atrium showed a considerably wider range of variation. While the arterial samples varied no more than 0.4 volume per cent from the original value, those from the atrium varied more than this in 20 per cent of the cases studied. Here, as in the arterial samples, there was no relationship of the variations to time, nor did the values show any consistent tendency to rise or fall as serial specimens were obtained.

The 25 comparative studies on ventricular blood gave results quite similar to those obtained with atrial blood. Approximately 20 per cent varied more than 0.4 volume per cent.

We believe that these variations in atrial and ventricular blood are more than technical errors, and represent either an actual change in cardiac output or incomplete mixing of blood in the heart chambers.

The possibility exists that actual variation in the cardiac output from minute to minute might account for some of the variations in the oxygen content of serial ventricular samples and also for the discrepancies between consecutive samples of ventricular and atrial blood. As far as can be determined from examination of ballistocardiograph tracings, variations of a magnitude necessary to account for these discrepancies in oxygen content of successive samples may occur at times, but a comprehensive study has not been made.

The right atrium receives blood containing widely variable amounts of oxygen. The blood draining from the kidneys has a high oxygen content, while that from the coronary circulation has a very low oxygen content. Thus streams of blood of different oxygen content may exist in the atrium, and may even persist in the ventricle. The observations reported here give no information as to the variation of oxygen content in blood collected from various places in the heart chambers. As far as possible the samples were taken with the catheter tip in the same location for the successive samples. However, with an extremely flexible catheter there may be considerable movement of the catheter tip with the beating of the heart and serial samples may contain relatively more blood from one area than another. On occasions the angulation of the catheter tip may become turned in the opposite direction from that seen on fluoroscopic examination.

The comparative studies of atrial and ventricular blood showed considerable variation. The variation is apparently random, there being no apparent consistent relationship between atrial and ventricular samples. From these data it would not appear to be possible, knowing one, to calculate the other. The variation in a similar study reported by Cournand and his colleagues (1) is less for reasons that we are unable to explain. One must remember that in both series the atrial sample was obtained on withdrawing the catheter from the ventricle. Hence the position of the catheter tip probably was closer to the tricuspid orifice than might be expected if the catheter were merely inserted into the atrium and the ventricle not entered. It is possible that the blood collected

from the right atrium in instances in which the ventricle is not entered is even less representative of ventricular blood.

The variations in the values for oxygen consumption are probably partly technical and partly real. The technical errors do not appear to result from the actual analysis of the respiratory gases, but occur from leaking around the mouth-piece, errors in measuring the volume of the expired air, leakage in the Douglas bag and variations in respiration produced by apprehension from breathing into a closed system. With the 2 or 3 minute sample errors also may result from starting and stopping the collection in different phases of the respiratory cycle. We have no data on the minute to minute variation in oxygen consumption in trained subjects.

The catheter method is more accurate in situations in which the arteriovenous oxygen difference is great. An error in arteriovenous difference of 0.4 volume per cent causes a much greater error in calculation of the cardiac output with an arteriovenous difference of 4.0 than it does with an arteriovenous difference of 8.0 volumes per cent (3).

A concise mathematical statement of the potential error is difficult because of the large number of variables. The catheter method represents a real advance in the tools available for the study of the cardiac output, but the errors in the method are great enough that any one set of observations may not give the actual cardiac output. The errors seem to be random, rather than systematic, and for this reason it is believed that the results with this method are valid when groups, rather than individuals, are studied.

SUMMARY AND CONCLUSIONS

1. An evaluation of the sources of error in the catheter method for determining the cardiac output utilizing the Fick principle was made.

2. There was little variation in oxygen content of consecutive samples of arterial blood. The maximum variation was 0.4 volume per cent.

3. Pairs of samples of atrial blood were collected without moving the catheter in 111 instances. The oxygen content of 78 per cent of these varied no more than 0.4 volume per cent; that of 22 per cent varied as much as 2.3 volumes per cent.

4. In 25 instances similar studies of the oxygen content of ventricular blood were made.

5. In 19 instances a comparison was made between the oxygen content of atrial and ventricular blood. In 11, the variation was no more than 0.4 volume per cent. In 17, it was no more than 1 volume per cent. In 2, it was 1.2 volumes per cent.

6. Forty-two duplicate samples of expired air were obtained. Thirty-one duplicate determinations checked within 10 per cent, whereas the remaining 11 varied as much as 20 per cent.

7. The errors in the method seem random rather than systematic. The catheter method for measuring the cardiac output is a useful procedure, but the errors

are sufficiently large so that the values in one set of determinations may not represent the actual cardiac output.

Miss Maurine Giese, Miss Eloise Cavin, Mrs. Jane Bailey and Miss Lois Jackson gave valuable technical assistance.

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THE BENEFICIAL EFFECTS OF YEAST ON THE BODY AND GONADAL WEIGHT OF IMMATURE RATS FED ALPHA-ESTRADIOL

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A number of reports have appeared concerning the rôle of B vitamins in the metabolism of estrogens. Livers of rats on vitamin B-deficient diets failed to inactivate estrone and other estrogenic substances although this ability was restored to normal if yeast (Biskind and Biskind, 1942) or thiamine and riboflavin (Segaloff and Segaloff, 1944; Singher et al., 1944) were subsequently administered. Furthermore, data are available indicating that estrogen administration may actually increase body requirements for B factors (Palmer and Hughes, 1942). In the present experiment, alpha-estradiol was fed to immature rats maintained on rations varying in B vitamin content, and the effects of feeding on body and gonadal weight determined.

PROCEDURE AND RESULTS. Three basal rations were employed in the present experiment: diets A, B and C. Diets A and B were purified rations containing the B complex factors in synthetic form and differing only in the level at which these vitamins were administered. Diet C was similar in composition but contained yeast in place of the synthetic B factors. All three rations were supplemented with 0.0, 0.2, 1.0 and 5.0 mgm. of alpha-estradiol per kgm. of diet¹. All rats received the following daily supplement: commercial oil (Mazola) 500 mgm., alpha-tocopherol 0.5 mgm., and vitamin A-D concentrate² containing 50 U.S.P. units of vitamin A and 5 U.S.P. units of vitamin D.

Ninety-one female and thirty male rats of the U.S.C. strain were employed in the present experiment. At twenty-one to twenty-three days of age litter mates were divided as far as possible among the experimental groups listed in table 1. Animals were kept in metal cages with screen bottoms to prevent access to feces, and sufficient food was administered to assure *ad lib* feeding. Feeding was continued for sixty days.

The findings are summarized in table 2. Rate of growth and gonadal weight were significantly reduced in rats maintained on synthetic diets containing 5 mgm. of alpha-estradiol per kgm. of diet (A4, B4). Substitution of yeast for the synthetic B vitamins resulted however in a significant increase in body and

¹ The alpha-estradiol for these experiments was kindly provided by Dr. Erwin Schwenk of the Schering Corporation, Bloomfield, New Jersey. One milligram alpha-estradiol \approx 12,000 R.U. or 120,000 I.U. estrone.

² Nopco fish oil concentrate, assaying 800,000 U.S.P. units of vitamin A and 80,000 U.S.P. units of vitamin D per gram.

gonadal weight (C4). No consistent reduction in body and gonadal weight was observed at lower levels of alpha-estradiol on any of the rations employed. Within seventy-two hours of the start of the dietary regime, vaginas opened in all rats fed estrogen-containing diets indicating that even the lowest level of alpha-estradiol was sufficiently high to exert an estrogenic effect under the conditions of the present experiment. With the exception of rats on diet B4 all groups made consistent gains in body weight. On diet B4, however, male and female rats failed to gain and even lost weight during the first two weeks of the dietary regimen, a loss of weight that could not be accounted for on the basis of

TABLE 1
*Composition of experimental diets**

DIETARY COMPONENT	DIETS A1, A2, A3, A4	DIETS B1, B2, B3, B4	DIETS C1, C2, C3, C4
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Casein†.....	22.0	22.0	22.0
Sucrose.....	73.5	73.5	63.5
Salt mixture‡.....	4.5	4.5	4.5
Yeast§.....	0.0	0.0	10.0

Vitamin supplements added to diets

	<i>mgm. per cent</i>	<i>mgm. per cent</i>	
Thiamine hydrochloride.....	0.2	20.0	0.0
Riboflavin.....	0.4	40.0	0.0
Pyridoxine hydrochloride.....	0.2	2.0	0.0
Calcium pantothenate.....	3.0	20.0	0.0
Choline chloride.....	120.0	120.0	0.0
2-methyl-naphthoquinone.....	0.2	0.5	0.0

* Alpha-estradiol was added to the diets as follows: A2, B2 and C2, 0.2 mgm. per kgm. of diet; A3, B3 and C3, 1.0 mgm. per kgm. of diet; A4, B4 and C4, 5.0 mgm. per kgm. of diet.

† Vitamin Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

‡ Salt mixture no. 1 (Sure, 1941).

§ HI-RIBO 24, Anheuser-Busch, Inc., St. Louis, Mo. Each gram contained the following vitamin potencies according to the manufacturer: thiamine 2 mgm., riboflavin 4 mgm., pyridoxine 100-170 micrograms, pantothenic acid 200-250 micrograms and nicotinic acid 400-500 micrograms.

decreased food intake. Subsequent to the second week growth was resumed and continued through the experimental period. During the sixth week of feeding a thinning of hair and areas of alopecia were observed in approximately one-third of the rats fed synthetic diets containing 5 mgm. of alpha-estradiol per kgm. of ration. These phenomena were not observed on diets containing lower levels of estrogen or in animals maintained on yeast-containing rations.

Animals were autopsied on the sixtieth day of feeding. Ovaries and testes were weighed, fixed in 10 per cent formol and sections prepared and stained with hematoxylin and eosin. With the exception of the gonads and related sexual structures, the gross appearance in all cases was essentially that of a well nour-

ished normal rat. No significant differences in weight or gross appearance were observed in the pituitary, adrenals, kidneys or heart in any of the groups involved. Ovarian weight was significantly reduced in rats fed synthetic rations

TABLE 2

Effects of feeding alpha-estradiol on the body weight and gonadal weight of immature rats fed different levels of the synthetic B vitamins or yeast in addition to basal diet

GROUP	ALPHA- ESTRADIOL PER KGM. RATION	NO. OF RATS	AVERAGE BODY WEIGHT		AVERAGE GONADAL WEIGHT†	RATIO OF GONADAL WEIGHT TO BODY WEIGHT × 10 ⁻³
			Initial	On 60th feeding day*		
Female rats						
	mgm.		gram	gram	mgm.	
A1	0.0	7	42.6	176.2 ± 8.7	52.1 (41-61)	0.30
B1	0.0	7	38.1	177.4 ± 5.7	56.0 (50-67)	0.32
C1	0.0	7	37.2	168.5 ± 10.8	56.3 (46-67)	0.33
A2	0.2	7	39.4	163.4 ± 6.8	46.7 (32-60)	0.29
B2	0.2	7	38.7	169.1 ± 7.9	48.1 (41-59)	0.28
C2	0.2	7	38.1	165.3 ± 2.8	56.4 (50-63)	0.34
A3	1.0	7	42.3	159.9 ± 4.1	52.7 (42-63)	0.33
B3	1.0	7	40.9	159.7 ± 10.6	44.0 (22-62)	0.28
C3	1.0	7	40.3	172.9 ± 6.7	56.1 (42-83)	0.32
A4	5.0	8	44.5	140.6 ± 4.2	24.3 (17-40)	0.17
B4	5.0	11	42.9	128.7 ± 3.9	21.3 (16-42)	0.17
C4	5.0	9	38.8	151.3 ± 3.2	38.4 (28-56)	0.25
Male rats						
				gram		
A1	0.0	3	43.5	247.3 ± 8.4	2.49 (2.41-2.52)	10.08
B1	0.0	3	38.4	236.9 ± 7.8	2.41 (2.38-2.47)	10.17
C1	0.0	3	41.3	222.4 ± 11.2	2.43 (2.39-2.85)	10.94
A3	1.0	3	38.0	214.0 ± 9.0	2.17 (2.04-2.28)	10.14
B3	1.0	3	38.7	195.6 ± 10.3	2.14 (1.52-2.65)	10.92
C3	1.0	3	38.6	222.7 ± 6.7	2.40 (2.20-2.52)	10.76
A4	5.0	4	43.1	162.7 ± 10.6	1.34 (0.80-2.08)	8.22
B4	5.0	4	40.3	147.5 ± 11.8	1.11 (0.39-2.09)	7.50
C4	5.0	4	41.5	203.1 ± 6.3	2.10 (1.80-2.49)	10.34

* Including standard error of the mean calculated as follows: $\sqrt{\frac{\sum d^2}{n}} / \sqrt{n}$ where

"d" is the deviation from the mean and "n" is the number of observations.

† The figures in parentheses represent the range of the individual values.

containing 5 mgm. of alpha-estradiol per kgm. of diet (A4, B4). Histologically the ovaries resembled those of immature rats twenty to thirty days of age. Mature follicles and corpora lutea in general were absent; immature follicles

were numerous and many young follicles were observed undergoing atresia. Similar findings were observed in three rats on diet B3 although ovaries appeared normal in the remainder of this series as well as on diet A3 and synthetic rations containing lower levels of alpha-estradiol. Histologically the ovaries appeared normal at all levels of alpha-estradiol when yeast was administered in place of the synthetic B vitamins.

The beneficial effects of yeast on the gonads of estrogen-fed rats were similarly demonstrated in the male. The testes of rats fed synthetic rations containing 5 mgm. of alpha-estradiol per kgm. of diet were significantly reduced in weight (table 2) and resembled histologically those described by Zondek in the estrogen-treated rat (Zondek, 1941). Spermatozoa were absent and various degrees of testicular injury were observed with some animals exhibiting but a single layer of cells in the seminiferous epithelium. Substitution of yeast for the synthetic vitamins resulted in a significant increase in testes weight and a microscopic picture similar to that of litter mates on estrogen-free diets. The testes appeared normal in rats maintained on rations containing lower levels of alpha-estradiol.

Discussion. This is the first report to our knowledge concerning the effects of estrogen-feeding to rats maintained on synthetic diets. Results indicate that rate of growth and gonadal weight were markedly depressed on synthetic rations containing 5 mgm. of alpha-estradiol per kgm. of diet but that substitution of yeast for the synthetic B vitamins exerted a protective effect on body and gonadal weight. The protective properties of yeast were not due to any of the B vitamins present in the synthetic rations. This is indicated by the fact that body and gonadal weight on diet B4 did not differ significantly from that observed on diet A4 although it contained 100 times as much thiamine and riboflavin, 10 times as much pyridoxine and $6\frac{2}{3}$ times as much calcium pantothenate as was present in the latter. Furthermore the amount of these vitamins in diet B4 was not less than that present in the yeast-containing ration C4 although no protective effect on body and gonadal weight was observed in the former series.

In addition to thiamine, riboflavin, pyridoxine, choline and pantothenic acid, yeast contains significant quantities of nicotinic acid, inositol, para-aminobenzoic acid, biotin, folic acid and presumably other as yet unidentified vitamins in addition to protein and ash. On diets free of alpha-estradiol rats on synthetic rations A and B synthesized sufficient quantities of these factors to meet requirements for growth and reproduction as well (Ershoff, 1945). The addition of 5 mgm. of alpha-estradiol per kgm. of diet resulted however in depression of body and gonadal weight and various degrees of gonadal pathology preventable in part by substitution of yeast for the synthetic B vitamins. These findings suggest that the above effects were due to nutritional deficiencies induced by estrogen-feeding under the conditions of the present experiment. It is not unlikely that the high levels of alpha-estradiol employed above increased body requirements for nutrients present in yeast but not present in sufficient amount in the synthetic diets employed nor synthesized in sufficient quantity by the intestinal flora or the animal's own tissues.

Considerable evidence is available to indicate that retardation of growth and depression of gonadal weight in estrogen-treated rats is due to impaired secretion of pituitary hormones since growth hormone and pituitary gonadotropins will maintain the body and gonadal weight of estrogen-treated rats (Zondek, 1941). The present experiment indicates that response to estrogens may be significantly altered by changing the composition of the basal ration although in the absence of exogenous estrogen body and gonadal weight were normal on the rations employed.

SUMMARY

Male and female rats were placed at weaning on purified rations containing 0.0, 0.2, 1.0 and 5.0 mgm. of alpha-estradiol per kgm. of diet. Three basal rations were employed. In two of the rations B vitamins were administered as synthetic factors; in the third they were present as yeast.

Rate of growth was significantly reduced in rats maintained on synthetic rations containing 5 mgm. of alpha-estradiol per kgm. of diet. Substitution of yeast for the synthetic vitamins in the above rations resulted in a significant increase in body weight. No adverse effects on body weight were noted at lower levels of alpha-estradiol.

A significant reduction in gonadal weight was observed in rats maintained on synthetic rations containing 5 mgm. of alpha-estradiol per kgm. of diet, ovaries remaining infantile both in weight and microscopic appearance and testes revealing a profound depletion of germinal epithelium. Substitution of yeast for the synthetic vitamins resulted in a significant increase in gonadal weight and the maintenance of normal histological structure. No adverse effects on gonadal weight or appearance were noted at lower levels of alpha-estradiol.

The suggestion is made that some factor in yeast other than the vitamins present in the synthetic rations may be responsible for the above effects.

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THE RELATIONSHIP OF CARDIAC GLYCOGEN DEPOSITION TO BLOOD KETONE LEVELS IN EXPERIMENTAL KETOSIS

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Following pancreatectomy, the glycogen content of the heart of the dog (1) and cat (2) has been shown to increase while that of the liver and skeletal muscles decreases. Recently we (3) have shown similar changes in glycogen deposition to occur in rats made diabetic by alloxan and suggested that the high cardiac glycogen may be associated with either the hyperglycemia or the hyperketonemia occurring in diabetes mellitus. Evans and Bowie (2) found no decrease in cardiac glycogen when the blood sugar was lowered by phloridzin. It has also been found (4), (5) that glucose feeding alone did not raise cardiac glycogen, but glucose plus insulin did if hypoglycemia did not occur. However, Bogue et al. (6) concluded from studies on heart-lung preparations that glucose was the source of cardiac glycogen. Insulin was not considered in this latter report. It therefore seems unlikely that the high cardiac glycogen in diabetes is due to the hyperglycemia. A high cardiac glycogen in fasted animals has been reported (4), (5), and since a ketosis occurs in fasted animals as well as in diabetic animals, the present work was undertaken with a view to investigating the relationship of cardiac glycogen deposition to ketosis.

METHODS AND RESULTS. Five series of adult male rats were kept on such dietary regimes that each would have a different blood ketone level. The animals in the five series were treated as follows: series 1, a stock diet ad libitum; series 2, fasted for 24 hours; series 3, fasted for 72 to 120 hours; series 4, no food for 72 to 120 hours except 460 to 690 mgm. of butyric acid (as sodium butyrate) per day administered by stomach tube in two daily doses; series 5, no food for 72 to 120 hours except glucose by stomach tube in amount calorically equivalent to the butyric acid given series 4. In all cases of fasting, food was removed from the cage in the morning and the duration of the fast measured from that time.

At autopsy, animals were injected with sodium pentobarbital, which has been shown to have no effect on the glycogen content of the tissues (7), and while anesthetized, a sample of blood for ketone and glucose determinations was taken. A lobe of the liver, the entire heart, and a portion of the hamstring muscles were removed, cut into small pieces with scissors, and immersed in hot potassium hydroxide solution for glycogen determinations. The entire procedure for the three tissues was completed in less than one minute. Determinations of blood sugar, blood ketones, and tissue glycogen were made by the methods cited in an earlier publication (3).

Table 1 summarizes the data on tissue glycogen, blood ketones and blood sugar. The first four series show a progressive increase in cardiac glycogen

directly correlated with the blood ketone levels. There is no relationship between cardiac glycogen and blood sugar. The liver and skeletal muscle glycogen is lowered in all series where the cardiac glycogen is increased. In the fifth series, glucose prevented the marked ketonemia associated with fasting and sodium butyrate feeding and also prevented the excessive storage of cardiac glycogen.

The glycogen content of the heart in the rats fasted for 24 hours is not as high as expected, considering the blood ketone level of 17.6 mgm. per cent. It occurred to us that the ketonemia was of such short duration that it had not had

TABLE 1

Tissue glycogen, blood sugar and blood ketones in rats under varied dietary conditions

SERIES	NO. OF ANIMALS	GLYCOGEN* MGM. PER 100 GRAMS TISSUE			KETONES mgm. per 100 ml.†	SUGAR mgm. per 100 ml.‡
		Liver	Heart	Skeletal muscle		
1. Normal fed	20	3056 ± 317†	442 ± 24†	515 ± 32†	3.2	136
2. 24-hr. fast	17	183 ± 32	502 ± 26	396 ± 28	17.6	115
3. 72-120-hr. fast	18	546 ± 85	708 ± 42	322 ± 21	26.3	125
4. 72-120-hr. sodium butyrate fed	14	421 ± 67	795 ± 28	366 ± 16	36.9	163
5. 72-120-hr. glucose fed	11	1982 ± 24	583 ± 27	414 ± 51	8.9	132

* As glucose.

† Standard error.

‡ As hydroxy-butyric acid.

TABLE 2

Blood ketones through a 24-hour fast

Hours without food.....	0	12	18	24
Blood ketones* Mgm. per 100 ml....	3.8	6.4	6.9	16.6

* As hydroxy-butyric acid. Each value represents the average of determinations on 10 animals.

time to affect the cardiac glycogen if such a relationship does exist. Therefore, a group of rats was fasted and ketone levels followed for 24 hours. As seen in table 2, the major increase in blood ketones during a 24-hour fast occurs after 18 hours.

Since a recent report by Capraro and Milla (8) indicates that certain ketone bodies may be converted in the body to lactic acid, it seemed desirable to know whether the rise in blood ketone levels is accompanied by increased blood lactic acid. Accordingly, lactic acid determinations were made on a number of rats treated as in series 1, 2, 3, and 4 as described above. Determinations were carried out by the photometric method of Hoffman (9). The data are given in

table 3. These values, when considered together with those for cardiac glycogen and blood ketones in table 1, fail to reveal any consistent relationship.

DISCUSSION. Numerous studies have been made both on perfused hearts and on intact animals, indicating a capacity of the myocardium to oxidize ketone bodies, but apparently the question of transformation of ketones to glycogen in the heart has been little discussed. Waters, Fletcher and Mirsky (10) found that beta hydroxy-butyric acid is utilized by heart-lung preparations of the dog, but that such utilization is decreased by adequate available glucose. Barnes et al. (11) found the optimum concentration for utilization of beta hydroxy-butyric acid by the heart-lung preparation to be 50 to 100 mgm. per cent, at which concentration 82 per cent of the metabolism may be at the expense of the ketones. Other workers have demonstrated the capacity of the heart to utilize fats, but Cruickshank and Startup (12) interpret their findings on perfused diabetic dog hearts to mean that transformation of fat to sugar does not occur. Cruickshank and Kosterlitz (13) produced a viable aglycemic rat heart and demonstrated utilization of fatty acids by the heart. They conclude, however, that the power of the heart to utilize fatty acids is only exercised when carbohy-

TABLE 3
Blood lactic acid levels in various dietary states

DIETARY REGIMEN	STOCK DIET UNFASTED	24-HOUR FAST	96-120-HOUR FAST	SODIUM BUTYRATE FED 72-120 HRS.
Blood lactic acid. Mgm. per 100 ml..	28.2	18.9	30.7	17.6

Each value represents the average of determinations on 10 animals expressed in mgm. per 100 ml.

drate sources are depleted to an extreme extent. Fletcher and Waters (14) interpret their results on the heart-lung preparation of the dog to mean that fat metabolized by the heart is derived from the fat stores of the organ itself rather than from the perfusing blood.

While our data do not prove that cardiac muscle converts ketone bodies to glycogen, they do indicate that conditions leading to ketosis result in increased storage of glycogen by the heart and that the amount of glycogen stored by the myocardium tends to parallel the ketone level of the blood. This suggests the possibility that the ketone bodies may be the source of the glycogen. It is noteworthy that the various dietary conditions used to bring about ketosis are those shown by Best, Haist and Ridout (15) to result in lowered insulin content of the pancreas and presumably lowered insulin content of the body tissues generally. According to these investigators, the insulin of the pancreas, lowered by fasting, is lowered still further by diets consisting wholly of fats. Since a more pronounced ketosis is also elicited in the latter condition, the increased glycogen storage might with equal reason be attributed to either the blood ketones or the lowered insulin or both.

Shorr (16) and co-workers conclude on the basis of studies on isolated tissues that the heart has the ability to resynthesize lactic acid, but no analyses for glycogen were made. Studies carried out on the heart-lung preparations by Bogue et al. (6) show no formation of cardiac glycogen from lactate added to the perfusion fluid. Our data do not indicate any relationship whatever between the amount of lactic acid in the blood and the storage of glycogen by the myocardium.

If the heart is capable of the conversion of ketone bodies to glycogen, this represents a capacity not exhibited by the liver. Deuel (17) and co-workers found the liver incapable of converting butyric acid to glycogen in either fed or fasted rats, and our data show in general an inverse relationship between liver or skeletal muscle glycogen storage and blood ketone levels.

SUMMARY

Five series of rats were so treated that each would have a different blood ketone level. Blood sugar, ketones, and lactic acid determinations were made, and the liver, heart and skeletal muscle were analysed for glycogen content.

No correlation is observed between cardiac glycogen and blood sugar or blood lactic acid, but a direct correlation is found between the blood ketone level and cardiac glycogen.

The possibility of cardiac glycogen being derived from ketone bodies is discussed.

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THE REFLEX ACTIVATION OF THE VASODILATOR FIBERS OF THE DORSAL ROOTS AND THEIR RÔLE IN VASODILATOR TONE^{1,2,3}

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Depressor reflexes, such as those elicited by depressor nerve stimulation, may cause peripheral vasodilatation by means of one or both of two reciprocal processes, activation of either sympathetic or dorsal root dilator fibers, and inhibition of sympathetic vasoconstrictor elements. Since many vasodilator and vasoconstrictor reflexes persist in completely sympathectomized animals (1, 2, 3, 4), it has been presumed that they are mediated by the remaining dorsal root dilator system. Validation of this assumption requires the demonstration that the reflex response disappears after complete removal of the dorsal root dilator system. The present experiments which were designed to serve this purpose consisted in the removal of the possibility of reflex activation of the dorsal root dilator fibers. Furthermore, the maintenance of vasoconstrictor tone is known to be a characteristic function of the sympathetic system and it would seem plausible, therefore, that an analogous vasodilator tone is maintained by the dorsal root vasodilator system, especially in view of the reciprocal action on other smooth muscle of the parasympathetic and sympathetic divisions of the autonomic system. Studies of the maintained blood pressure were therefore made after removal of both the dorsal root vasodilator system and the sympathetic vasoconstrictor system from tonic reflex activation to provide direct information as to the importance of these two systems in the maintenance of vascular tone.

METHODS. Cats were lightly anesthetized with intraperitoneal injections of nembutal or delvinal. Laminectomies were begun at about 3S or 4S and continued cephalad from there to 4C, the cord being kept moist and warm with hot, wrung out saline packs over which was placed a long rubber tube connected to a hot water tap and the whole area closed in by clamping the overlying skin incision. The cord was prepared by slitting the dura for five segment intervals at a time, beginning at the sacral region and then passing on to the next group of segments. A long loop of silk thread was placed on the dorsal surface of the cauda equina and the thread run between the dorsal and ventral roots on each side, using small curved needles. After the 3C roots had been thus separated, the ends of the threads were led out of the cephalad end of the incision. Pulling both ends of the thread in a caudad direction separated all the dorsal roots from the cord.

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In order to perform the sympathectomy all the ventral roots on both sides between 7C and 2L were loosely ligated separately just outside their exit from the spinal column. After such ligation, pulling all the threads connected to the ventral roots simultaneously deprived the sympathetic system of any reflex activation.

The animal was next placed on its back in a special cradle so arranged that the threads from the incision could be pulled from under the raised cradle. The central end of the depressor nerve was prepared for stimulation and the femoral artery cannulated to record blood pressure. The usual procedure involved stimulation of the depressor nerve centrally by means of a thyatron stimulator with weak current at 2-5 shocks per second and recording of the corresponding reflex depression in the blood pressure record. The dorsal roots were then cut, the stimulation repeated and the effects recorded. This was followed by sympathectomy, final stimulation, and recording.

RESULTS. Satisfactory experiments were performed on five out of the twenty-three cats used. The maintained blood pressures of these five preparations before any root resections were attempted, ranged between 100 and 160 mm. Hg. Stimulation of the depressor nerve resulted in a rather slow, but well defined, decrease in blood pressure ranging from 15 to 45 mm. Hg. Following resection of all the dorsal roots from the cord, the pressure rose and was maintained at from 20 to 40 mm. Hg above the starting values. Subsequent stimulation of the depressor nerve failed to produce any change in the blood pressure despite the fact that the vascular system was still innervated by the sympathetic system. Following this, resection of the sympathetic system from the spinal cord resulted in a decrease of pressure ranging from 40 to 60 mm. Hg following which the blood pressure became stabilized at this new level, which varied from 90 mm. Hg in the various preparations. Stimulation of the depressor nerve again failed to alter these values.

DISCUSSION. The definite and sustained rise in blood pressure seen in all of these experiments suggests that normal vascular tone may be assumed to represent the resultant of opposing vasoconstrictor tonus of the sympathetic system and vasodilator tonus of the dorsal root vasodilator system. Total dorsal rhizotomy upsets this balance in favor of the sympathetic vasoconstrictor system and results in an increase in the blood pressure. The experimental results reported here support the concept which was derived from the fact that completely sympathectomized animals can maintain a normal blood pressure as reported by Rosenbluth and Cannon (14), Bacq et al. (15), Leriche and Fontaine (16), Bradford Cannon (17), and McAllister and Root (18) which has never been refuted and which presumably indicates the result of almost complete reflex inhibition of the vasodilator center and its motor pathway through the dorsal roots. This reflex inhibition may be increased because of the lack of the previously opposed sympathetic vasoconstriction.

This view is further supported by studies on paw vasodilatation in various animals. It has been shown that sympathectomized paws always exhibit a maintained high temperature in the case of the cat by Hinsey (19) and in the monkey by Zuckerman and Ruch (20). The latter workers also showed that when the

foot of the monkey remained connected with the central nervous system only by the dorsal roots, it always exhibited a higher temperature than did the normal foot, indicating unopposed dilator tone. Deafferentation causes the temperature of the paw to fall below that of the normal paw. This was shown for the monkey by Zuckerman and Ruch (20), for the dog by Werzilloff (21) and for the cat by Hinsey (19).

The work of Bozler (22) and Karlik (23) indicates that blood vessels dilated by stimulation of the vasodilator fibers of the dorsal roots can be returned to their normal size if the sympathetic supply is stimulated at its normal rate, providing good evidence that these two systems may tonically oppose each other.

The failure to obtain any change in blood pressure on depressor nerve stimulation after all dorsal roots have been eliminated provides direct evidence that they are an essential pathway for the excitation of the reflex vasodilatation. When the dorsal root fibers are intact, they are reflexly activated by stimulation of the depressor nerve, presumably via the vasodilator center of the medulla (1, 2). These results supplement those of Tournade and Malmejac (11) who were unable to obtain dilatation of the vessels of the paw following section of the dorsal roots supplying this member. Rosenblueth and Cannon (1) were able to obtain reflex increases and decreases in blood pressure in curarized, completely sympathectomized, vagotomized cats. Analogous results were found by Bayliss (2), Freeman and Rosenblueth (3), Bacq, Brouha and Heymans (4), and, in the human, by Gambill, Adson and Hines (8), all of whom conclude that an extra-sympathetic vasoregulatory system must exist.

Pinkston, Partington and Rosenblueth (7) have shown that reflex activation of dorsal root dilator fibers occurs following stimulation of afferent nerves in completely sympathectomized cats and dogs. Bishop, Heinbecker and O'Leary (10), and Bayliss (2) earlier, found that the vasodilatation following depressor nerve stimulation occurred to a greater extent in the sympathectomized limb than in the normal limb although Dole and Morison (9) have indicated otherwise.

These experiments also provide further evidence concerning the dispensability of the sympathetic system in the cat. Bacq, Brouha and Heymans (4) have indicated that completely sympathectomized cats still exhibit carotid sinus reflexes which are not found in sympathectomized dogs. Pinkston et al. (7) also have shown that completely sympathectomized cats exhibit much more obvious vasomotor reflexes than do similarly prepared dogs. Urey and Gellhorn (12) and Weinstein and Bender (13) have also shown sympathetic dispensability in the case of the pupil of the cat's eye. Variations in pupil size in this animal depend almost entirely upon parasympathetic excitation and inhibition. The experiments reported in this paper show that inhibition of the sympathetic plays a very minor, if any, rôle in reflex vasodilatation since there was no alteration in blood pressure as a result of depressor nerve stimulation despite the fact that the vascular system of the totally deafferented animal was still innervated by the sympathetic system.

Of great interest is the finding that the blood pressure returns to and remains at approximately the previous normal level following complete resection of the dorsal root dilator fibers and the sympathetic vasoconstrictor fibers. These

procedures presumably leave the vascular system completely denervated with the exception of the peripheral portions of the sympathetic and dorsal root dilator fibers. It is unlikely that these remaining portions could spontaneously discharge enough impulses to keep the vascular tonus and blood pressure at nearly normal levels. One explanation may be that the smooth muscle of the vascular system may exhibit a fairly high degree of idionus which the vasoconstrictor and vasodilator systems alter. Smooth muscle idionus has been indicated in the case of the iris by Gullberg, Olmsted, and Wagman (24) and for the gut and bladder by Henderson and Roepke (25, 26).

CONCLUSIONS

Evidence is presented to show that the vasodilator fibers of the dorsal roots can be reflexly activated following stimulation of the depressor nerve.

It is further indicated that these vasodilator fibers mediate impulses which are concerned with the maintenance of a vasodilator tone.

Further consideration is given to the hypothesis that the sympathetic system is highly dispensable in the cat.

It is also suggested that the smooth musculature of the vascular system exhibits idionus, the extent of which is altered by the antagonistic action of the sympathetic vasoconstrictor and dorsal root vasodilator fibers.

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THE PRODUCTION OF ACETYLCHOLINE IN ANTIDROMIC VASODILATATION^{1,2}

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It is a generally accepted fact that some sort of humoral agent is associated with the production of antidromic vasodilatation. There is some question as to the nature of the substance. Doi (1) showed that antidromic vasodilatation can be obtained in the web of the foot of the frog despite previous capillary dilatation induced by histamine, this suggesting that a different agent, presumably acetylcholine, is concerned in the production of the arteriolar vasodilatation. Wybauw (2, 3, 4) has indicated the cholinergic nature of the antidromal fibers since he showed that intravenous eserine potentiated antidromic vasodilatation in the dog whereas intravenous atropine weakened the response considerably. Furthermore, perfusates of the dilated area caused contraction of eserinated leech muscle, depressed the blood pressure of anesthetized cats, and decreased the rate of the frog heart. Gollwitzer-Meier and Otte (5) found that perfusates of the mesenteric and splenic vascular beds contained increased amounts of acetylcholine after carotid sinus stimulation. Gaddum and Kwiatowski (6) proved that acetylcholine was secreted during the antidromic vasodilatation of the rabbit's ear induced by stimulation of the peripheral end of the sectioned auricular nerves since perfusates of these dilated ears caused contraction of eserinated leech muscle. Hasama (7) showed that atropine perfusion of the toad leg depressed the extent of antidromic vasodilatation.

Some evidence has been presented suggesting that acetylcholine is not the responsible humoral agent in antidromic vasodilatation. Gollwitzer-Meier and Otte (5) indicated that perfusates of the dilated splenic and mesenteric vascular beds induced by dorsal root stimulation failed to show the presence of acetylcholine. Mosonyi (8), Reid Hunt (9) and Ranson (10) showed that antidromic vasodilatation in the cat was not prevented by atropine and Pietrowski (11) showed this to be true for the rabbit.

It seemed important to repeat the experiment of Gaddum and Kwiatowski on the ear of the rabbit somewhat more carefully since these workers mention the fact that the auricular nerve carries motor fibers which caused contraction of some muscles at the base of the ear and they noted that those contractions may have been the source of some of the acetylcholine which they demonstrated in the dilated areas. Furthermore, Marinesco (12) showed that some sympathetic

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fibers, derived in part from the cervical sympathetic, and in part from the stellate ganglia, traversed the auricular nerves.

METHODS. The third cervical dorsal root was avulsed from the cord in nembutalized rabbits. Attempts to perform a laminectomy in this region were unsuccessful, since they resulted in uncontrollable bleeding which made it impossible to dissect this root free. The peripheral stump of this root, sectioned central to the ganglion, was stimulated with weak shocks at 2.5 per second with a thyratron stimulator. Samples of blood were obtained from the auricular vein where it joins the external jugular vein. The vein was entered with a 27 gauge needle and the samples were drawn into a tuberculin syringe previously prepared to contain 0.05 cc. "Liquaemin" (heparin) and 0.02 cc. 0.01 per cent physostigmine salicylate. Control samples were withdrawn before stimulation and the test samples taken during the period of vasodilatation of the ear vessels resulting from stimulation of the dorsal root. These samples were then centrifuged and the plasma tested for the presence of acetylcholine. The effects of 0.2 cc. of plasma from the normal ear and 0.2 cc. plasma from the dilated ear were then tested on eserinizd frog hearts.

Ungar and Parrot (13) have reported the isolation of a substance from the blood of antidromically vasodilated areas which differs in its chemical nature from acetylcholine or histamine and they identify this substance with adrenoxine. This substance presumably destroys adrenalin and they ascribed the antidromic vasodilatation to the action of this substance. An attempt was made in the course of the experiments to be described to demonstrate the action of this substance. Samples from the antidromically vasodilated ear were incubated with adrenalin (1:1000) at 36°C. for three hours and the effect of this mixture was tested on the frog heart in an effort to determine whether the adrenalin was destroyed by any adrenoxine which might have been present.

RESULTS AND DISCUSSION. As indicated in figure 1, neither the addition of 0.5 cc. 1:10,000 physostigmine nor 0.2 cc. eserinizd plasma from the non-vasodilated ear affected the frog heart in any way. The addition of either 0.2 cc. of 1:1000 acetylcholine or 0.2 cc. eserinizd plasma from the antidromically vasodilated ear caused complete inhibition of the heart. Such responses were typical of the results obtained in five rabbits.

Stimulation of the peripheral stump of the avulsed third cervical dorsal root resulted in vasodilatation of the ipsilateral ear of the rabbit as well as the appearance of a substance in the venous blood of the ear which would inhibit the beating of the eserinizd frog heart in a manner identical with that of acetylcholine.

It is difficult to explain the fact that several reports have shown that atropine has no effect on antidromic vasodilatation in the face of equally convincing evidence that acetylcholine is present in the antidromically vasodilated areas. It may be possible that these particular sites of acetylcholine production are not readily accessible to circulating atropine. In any event, proof of the presence of acetylcholine is certainly more direct evidence of its production than is a demonstration of the absence of inhibition of the effect by atropine. Because of the continuance of the dilatation after cessation of the stimulation of the appropriate

dorsal root, it appears that either the production of acetylcholine continues after cessation of the stimulation, or more probably that another dilator substance, particularly histamine, may prolong the effect.

Histamine has been proposed by many workers (Ungar (14), Lewis and Marvin (15), Bena (16), Doi (1), Hara (17)) as the substance responsible for the antidromic vasodilatation. However, it has been shown in several experiments (Barsoum and Gaddum (18), Anrep et al. (19)) that almost any condition which results in circulatory stasis will also cause the production of histamine. For this reason reports of the presence of histamine in vasodilated areas should not

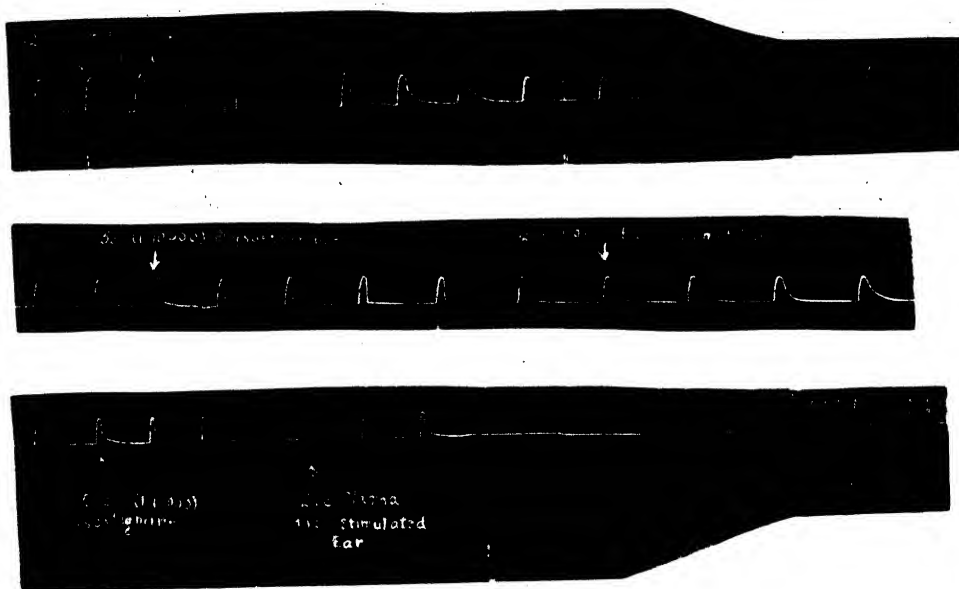



Fig. 1. Effects on an eserinizied frog heart of 0.2 cc 1:1000 acetyl choline, 0.2 cc plasma from the normal ear of a rabbit (no. 3), and 0.2 cc. plasma from an antidromically vasodilated rabbit ear (no. 3). It will be noted that acetylcholine and the plasma of the antidromically vasodilated ear both caused inhibition of the heart whereas plasma from the normal ear had no such effect.

necessarily be taken to mean that the histamine was the initial causal factor in the effect. It therefore becomes difficult to ascribe to histamine the rôle of initiating the vasodilatation; it is rather more probable, at least in the case of antidromic vasodilatation, that it may maintain or even increase the degree of vasodilatation initiated by some other mechanism and its production is incidental to the original vasodilatation.

Adrenalin incubated with plasma derived from the antidromically vasodilated ear retained the ability to increase the rate of beating of the frog heart as shown in figure 2. It is therefore concluded that the production of adrenoxine and its rôle in the production of antidromic vasodilatation are insignificant.

The results of these experiments confirm the findings of Wybauw and support his conclusions concerning the cholinergic nature of the fibers involved in antidromic vasodilatation. Such evidence raises the question of the distribution of parasympathetic fibers. It has always been assumed that these fibers were limited to their cranial and sacral outflow. Further evidence presented in the preceding papers of this series indicates that the parasympathetic-type fibers of the dorsal root synapse in the dorsal root ganglia with upper motor neurones of spinal or supra-spinal origin and that these fibers can be activated reflexly by stimulation of the depressor nerve. It has further been shown that they mediate impulses concerned in the maintenance of vasodilator tone (Bach (20), (21), (22)). These lines of evidence would point to the existence of a spinal outflow of parasympathetic fibers analogous to the sympathetic outflow.

A mechanism of activation of the antidromic vasodilator fibers of the dorsal root may now be postulated. Tonic stimulation of the depressor nerve by way of the carotid sinus causes discharges into the vasodilator center of the medulla and subsequent tonic discharges from this center travel down the spinal cord and



Injection of 1:1000 epinephrine incubated with the plasma from stimulated ear

Fig. 2. Effect on the frog heart of 0.1 cc 1:1000 epinephrine previously incubated with plasma from the antidromically vasodilated ear of the rabbit. This plasma apparently contained no substance which would interfere with the normal acceleratory effect of epinephrine.

pass out of the central nervous system by fibers which traverse the dorsal roots and make synaptic connections with neurones of the dorsal root ganglion. These latter lower motor neurones give rise to parasympathetic type fibers, reflex activation of which induces vasodilatation associated with the production of acetylcholine in the appropriate dermatome. Such vasodilatation may be maintained and even increased subsequent to cessation of stimulation by the action of histamine produced by the initial stasis.

CONCLUSIONS

Evidence is presented to show that acetylcholine is definitely formed as a result of stimulation of the vasodilator fibers of the dorsal roots in the rabbit and that adrenoxine is not produced in significant amounts.

The rôle of histamine in this and other types of vasodilatation is discussed and it is suggested that this substance is probably formed after the initiation of the vasodilatation and so is not the causal factor.

The mechanism of reflex activation and action of the parasympathetic type fibers concerned in antidromic vasodilatation is suggested.

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BLOOD HISTAMINE LEVELS IN EXPERIMENTAL BURNS

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Although ulceration of the duodenum is an accepted complication in burns, Harkins (1938) lists 28 possible theories regarding etiology. Recently one of us (Hartman, 1945a) reported that in a series of severely burned animals, dressed with vaseline or similar preparations, Curling's ulcer occurred in 63.3 per cent, whereas only 6.6 per cent of a group, burned to the same degree but dressed with tannic acid or other tanning agents, developed duodenal ulceration. These findings suggested that loss of plasma, autolysis with infection, and acidosis might be causative factors in the formation of Curling's ulcer. Further work on this problem (Hartman, 1945b) lends support to the hypothesis that infection is primarily concerned in the production of duodenal ulceration in burns. The incidence of ulceration fell from 77.7 per cent in a control group of burned animals, dressed with sterile vaseline, to 23 per cent in a group with similar dressings, but given daily injections of penicillin. These studies offered no confirmation of the hyperacidity theory as an explanation of the ulceration in burns. The author (Hartman, 1945a) did suggest that normal acidity may be sufficient to promote ulcer formation in the edematous, congested mucosa of the burned animal. The evidence in the literature is conflicting with the work of Necheles and Olsen (1941) in favor of, and with that of Wilson (1935) against the hyperacidity concept.

It has been established that a gradual and prolonged liberation of histamine from histamine-beeswax injections with the simultaneous copious gastric juice of maximal acidity will produce gastric and duodenal ulcers in animals (Hay, Varco, Code and Wangensteen, 1942). Since histamine is liberated at the site of a burn (Harris, 1927) one may speculate as to whether it plays a rôle in the stimulation of an acid gastric juice and thus in the formation of Curling's ulcer. Would the histamine be freed from the burned area in effective amounts, over a period of time sufficient to maintain a gastric acidity, which would promote the ulceration so often encountered in this series of burned animals? Seeking an answer to this question, the problem was approached from the standpoint of the blood histamine. The object of these experiments, then, has been primarily to determine the order of magnitude and duration of the blood histamine change following experimental burns, and secondly, to determine whether there is a correlation between the blood histamine equivalent and the gastric secretion in the severely burned animal.

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Clinical studies on burned patients show that there is an upward trend in blood histamine values following a burn. The first contribution on the subject was made by Barsoum and Gaddum (1936) on 9 patients with extensive cutaneous burns. A detailed description of 4 cases shows that there is a marked rise to 4 times the normal blood histamine level within 5 to 6 days following the burns. These high levels are maintained for 10 to 20 days. Code and MacDonald (1937) contribute one burn case which showed a steady rise in blood histamine from the first to the twelfth day, with a gradual return to the normal during the subsequent 2 months. Rose and Browne (1942) reported 7 severe burn cases. Three of these cases showed an elevated blood histamine equivalent within one hour after the burn. In all cases a rapid and marked decrease occurred on the third to the fifth day coincident with the development of edema and toxemia. As the edema subsided the histamine level rose to the normal and above the normal value. In 3 fatal cases these workers noted a marked decrease in blood histamine 12 to 36 hours before death. They feel that there may be a correlation between this fall in blood histamine and the shock due to trauma or burns.

Kisima (1938) found that cutaneous burns in dogs increased the histamine content of all tissues as well as that of the blood. The blood histamine showed an initial rise within 6 to 12 hours after burns, reaching its maximum within 48 to 96 hours. After 2 to 3 days this level gradually declined toward the normal in the succeeding 10 days. Immediate resection of the burned skin prevented the histamine rise in tissue and blood. Similar changes were observed in 4 clinical patients. Kisima used the physiological assay of Akiyama (1937) and the micro-colorimetric method of Yokoyama (1936). In a comparison of the two methods on blood, urine and tissue, the inaccuracy of the colorimetric method was demonstrated. The physiological assay of Akiyama is similar in principle to that of Code's modification of the Barsoum and Gaddum method although there are variations in detail. A lack of comparative data on these physiological assays makes an evaluation of Kisima's quantitative results difficult. Consequently, further study of blood histamine levels in burned animals is indicated from a purely academic point of view as well as its relation to the problem in hand.

METHODS. The histamine content of the blood was determined using Code's (1937a) modification of the Barsoum and Gaddum method (1935). Since it is a physiological assay the method was tested in duplicate on known histamine solutions and on normal dog blood specimens. Blood specimens were drawn rapidly from the jugular vein to minimize stasis and were immediately discharged into trichloroacetic acid. Blood histamine determinations were done on the experimental animals at varying intervals to determine the trend of the blood histamine change. All histamine determinations are expressed as gamma of histamine base per 100 cc. of blood ($\text{gamma} = 0.001 \text{ mgm.}$, or 1 microgram) (figs. 1 and 2).

All the animals studied were given a third degree burn over 50 per cent to 60 per cent of the body surface while under morphine or chloroform anesthesia. The dressings were of sterile vaseline and gauze, which were not changed. Every

effort was made to increase the survival time by special feeding and intravenous fluids. The survival time ranged from 8 to 12 days. Autopsies were performed in all cases. Gastric analyses, both fasting and after 150 cc. of 5 per cent alcohol, were carried out on a relatively small number of normal and experimental animals.

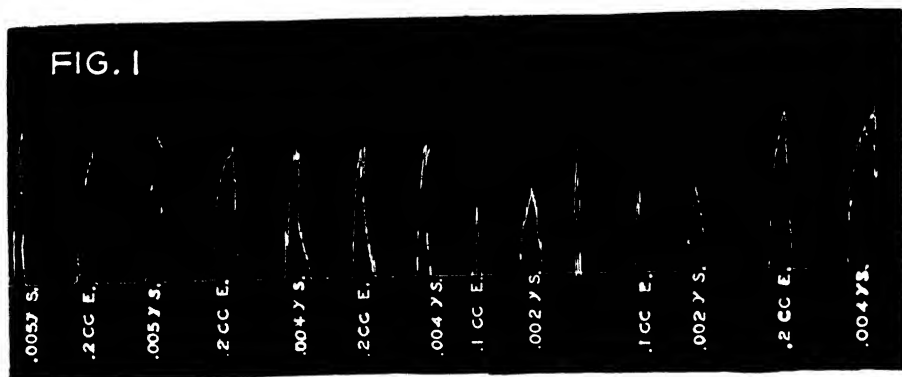


Fig. 1. Normal blood histamine = 2 gamma/100 cc.

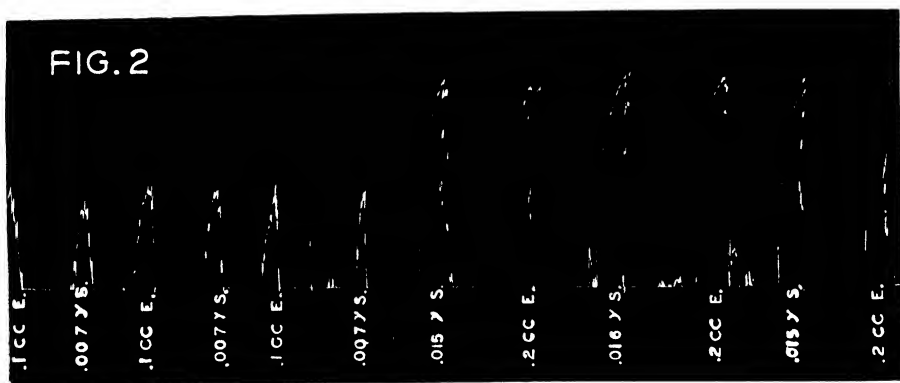


Fig. 2. Blood histamine 48 hours after burn = 7.5 gamma/100 cc.

Figs. 1 and 2. Histamine assay on dog blood extracts, using terminal ileum of the guinea pig.

Volume of muscle bath = 3 cc. Perfusion fluid = atropinized Tyrode's solution.

Histamine standard (S) in gamma is alternated with blood extract (E) in cc.

RESULTS. Blood histamine determinations were made in 9 normal and in 14 burned animals. Five of the animals, receiving daily injections of penicillin, showed no ulceration at autopsy, whereas 8 out of 9 untreated animals developed ulceration. Blood histamine levels were elevated in both groups. In fact, 13 out of 14 burned animals exhibited a rise in the blood histamine following burns, as shown in figure 3. These results show a blood histamine rise to 2.5 times the

normal level within 24 to 48 hours which continues to increase to about 5 times the normal value about the fourth day. The elevated blood histamine equivalent may be maintained for a few days, after which the level decreases toward the normal in the succeeding days.

In a series of 4 animals it was planned to determine the blood histamine on the day prior to the burn, 48 hours after the burn, and on the tenth day. In animal A the results obtained were 2 gamma/100 cc. as a normal value and the very low

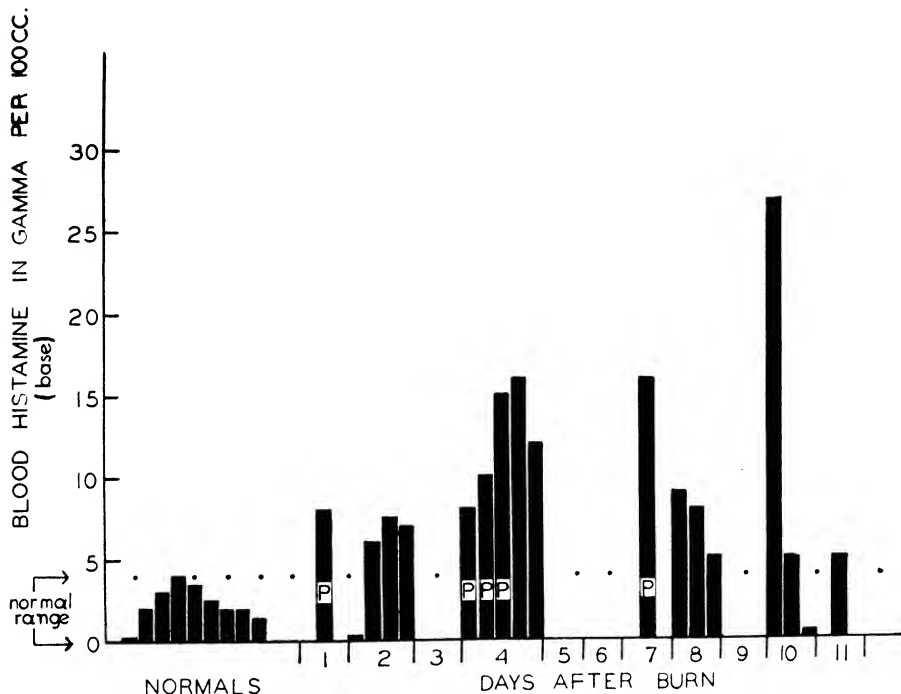


Fig. 3. Distribution of blood histamine values after experimental burns. Normal values ranging from 0.1 gamma to 4 gamma/100 cc. of blood on 9 animals are shown. Data from 14 burn animals, obtained between the first and eleventh day are depicted. Penicillin was administered daily to 5 animals marked (P).

figures of 0.1 gamma/100 cc. and 0.2 gamma/100 cc. on the second and tenth day samples. The animal died on the eleventh day, showing no signs of ulceration. In animal B the values obtained were respectively 3.5 gamma/100 cc., 7.5 gamma/100 cc., and 5 gamma/100 cc. of blood. At autopsy on the eleventh day multiple ulcers of varying size were observed. Animal C had a normal value of 3 gamma/100 cc. and a 48 hour value of 6 gamma/100 cc. The animal died as we were in the process of drawing the tenth day sample. The sample showed a blood histamine equivalent of 27 gamma/100 cc. Autopsy revealed a small ulcer with an 8 mm. crater filled with clotted blood. The stomach and small

bowel contained quantities of fresh and altered blood. The tissues were unusually pale, leading to the conclusion that death was due to hematogenic shock. Animal D possessed no demonstrable histamine in the normal blood sample, while the 48 hour specimen showed a rise to 7 gamma/100 cc. The animal was found dead on the morning of the tenth day and so no sample was available. At autopsy small gastric ulcers were found.

TABLE 1

A correlation of blood histamine values with gastric analyses and ulceration in experimental burns

ANIMAL NO.	TIME AFTER BURNS	BLOOD HISTAMINE	SPECIMEN	GASTRIC ANALYSES			GUAIC	AUTOPSY (INCIDENCE OF ULCERATION)
				Free HCl	HCl def.	Total acids		
D	days	gamma/100 cc.						
	11 12	5	Fasting	6		58	+++	Death—Pyloric region of stomach, ulcer 1 cm. in diameter; duodenum, multiple smaller ulcers
E	4 6	12	Fasting	2	0	12	++++	Death—small multiple ulcers
			15 min.	4	0	6	neg.	
			30 min.	4	0	8	neg.	
			45 min.	14	0	22	++	
			60 min.	22	0	30	++++	
			75 min.	8	0	22	++++	
	8							
F	4 6	16	Fasting	10	0	12	++++	Death—small ulcers
			15 min.	2	0	10	neg.	
			30 min.	0	4	2	neg.	
			45 min.	0	2	12	++++	
	8	9	Fasting	2	0	8	++++	
			15 min.	2	0	10	++++	
			30 min.	0	4	12	++++	
			45 min.	0	2	4	++++	
	10		60 min.	0	4	14		

Gastric analyses in a few of the experimental animals on which blood histamine determinations were made showed that both the free HCl and the total acid are either normal or reduced in spite of the increased blood histamine levels. The accompanying figures are illustrative of this fact (table 1).

DISCUSSION. Code's modification of the Barsoum and Gaddum method for the quantitative estimation of histamine in the blood yields values below 4

gamma/100 cc. in the normal dog. The average histamine value of 2.3 gamma/100 cc. of blood obtained in this study of 9 normal dogs agrees very well with the average value of 3.2 gamma/100 cc. in 14 estimations on 5 dogs obtained by Code (1937a). In some cases the histamine content of dog's blood cannot be accurately measured with the present methods. Code (1937b) shows this in a series of consistently low values in which he prefers to discard values lower than 1 gamma/100 cc. of blood as unreliable. Similarly, we place no significance on the low values of the order of magnitude of 0.1 to 0.2 gamma/100 cc. of blood.

The rise in blood histamine observed in these burned animals is in agreement with the clinical as well as the experimental data in the literature. It is difficult, however, to compare the magnitude and duration of the blood histamine increase in the post-burn days for there is a wide variation in the extent of the burns in the clinical and experimental data recorded. The blood histamine level began to rise in our experimental animals within 24 to 48 hours but did not reach a maximum until the fourth day, whereas Kisima found the maximum level within 48 to 96 hours. Since there is a direct relation between the area of the burn and the magnitude and duration of the histamine rise (Barsoum and Gaddum, 1937) the greater area burned in this study (50 per cent to 60 per cent of the body surface) as compared to the smaller area (10 per cent to 20 per cent of the body surface) in Kisima's dogs is undoubtedly the reason for the difference.

These results are not in complete agreement with the report of Rose and Browne (1942). First, these workers observed a decrease in blood histamine as toxemia and edema developed, yet neither of these conditions appeared in this experimental study. Secondly, they associate the marked decrease in blood histamine observed 12 to 36 hours before death with the shock due to severe burns. The blood histamine value of 27 gamma/100 cc. obtained in animal C is not in agreement with this concept. The fact that the animal was moribund from hematogenic shock is probably a factor in the height to which the histamine level rose. Thus, this is in keeping with the concept of an increased liberation of histamine-like substances in traumatic shock (Dale and Laidlaw, 1919).

The elevation in blood histamine in both the untreated burn animals and in the burn animals receiving penicillin was of the same magnitude (fig. 3). Therefore, the fact that increased blood histamine equivalents occur in dogs with and without ulceration of the duodenum indicates that blood histamine is not the significant etiologic factor in this condition.

In an evaluation of high blood histamine levels one should consider the distribution of the histamine within the blood. It is more or less accepted that histamine must be present in the plasma to be physiologically active, whereas it is inactive if fixed within the cells. Consequently, whole blood determinations alone, although indicative of the trends in histamine change, must be supplemented by figures showing the distribution between cells and plasma respectively. Code (1937b) has studied this in the normal blood of dogs and has found the histamine content of the plasma and red cells as low as 10 to 12 per cent with 70 to 100 per cent in the white blood cells. Considering this point we should not conclude that the increased histamine content of the blood observed in this study is proof that the physiologically active histamine is also elevated.

The results of gastric analyses on normal and burn animals showed a lack of hyperacidity in the burned animals. Histamine blood levels were elevated in the few animals on which both determinations were made. This indicates that the blood histamine level is either not of sufficient magnitude to stimulate gastric acidity or a large percentage of the histamine is bound within the cells in an inactive state. In either case the increased histamine blood level does not stimulate the endogenous mechanism of the stomach for acid production.

The lack of gastric acidity observed in this study is in disagreement with the work of Necheles and Olsen (1941). These workers concluded that the increased gastric acidity and motility associated with the diminished secretion of bile and pancreatic juice in the burned animal fostered the formation of Curling's ulcer.

Wilson (1935), on the other hand, has found no excessive gastric acidity in patients suffering from burns. Further support to our findings of normal or low gastric acidity in burned animals is afforded by the work of Dragsted (1945). He has studied the effect of severe burns on the gastric secretion through the use of isolated denervated pouches of the stomach in the dog. A 33 per cent body surface burn produced a marked and long continued suppression of gastric secretion and acidity as well as the complication of Curling's ulcer. Injection of histamine (1 cc. of 1:1000 histamine di-hydrochloride) in one of these burned animals produced a copious flow of gastric juice with a high free acidity. In a later experiment the exsanguinated blood of a burned dog (60 per cent of the body surface) was transfused into a control dog with a Heidenhair pouch, causing the typical suppression of gastric secretion in the recipient. Dragstedt feels that these data refute the concept that extensive burns stimulate gastric secretion in a manner comparable to that produced by histamine injections. The results of this study agree with the concept that the increased blood histamine liberated at the site of the burn does not seem to be sufficiently high in activity to cause an increase in gastric acidity and secretion.

SUMMARY

1. An increase in blood histamine was observed in 13 out of 14 animals burned over 50 per cent to 60 per cent of their body surface and dressed with sterile vaseline. Daily injections of penicillin in 5 of the animals had no effect on the rising blood histamine values. The blood histamine level rose to 2.5 times the average normal figure within 24 to 48 hours after the burn, with a maximum approximately 5 times the normal figure about the fourth day. After the sixth to seventh day the level decreased gradually toward the normal in the succeeding days.

2. Our findings show no positive evidence that the elevated blood histamine levels stimulate excessive gastric acidity or play a rôle in the formation of Curling's ulcer. The normal or subnormal gastric acidity titrations suggest that the blood histamine was either not elevated enough to produce hyperacidity or it was in an inactive form. Since penicillin treated animals did not develop ulceration in spite of elevated blood histamine values, there is further indication that blood histamine does not enter into the pathogenesis of Curling's ulcer.

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THE RENAL REABSORPTION OF AMINO ACIDS IN DOGS: VALINE, LEUCINE AND ISOLEUCINE¹

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At normal plasma concentrations the reabsorption of amino acids from the glomerular filtrate is practically complete and consequently the amounts excreted in the urine are relatively insignificant (Pitts, 1943; Goettsch, Lyttle, Grim, and Dunbar, 1944). Elevation of the plasma concentration to levels above normal by the administration of amino acids results in an increase in the rate of tubular reabsorption (Doty, 1941; Pitts, 1943, 1944; Goettsch et al., 1944). Reabsorption is somewhat less complete under these conditions, however, because the process fails to keep pace with the increased rate of amino acid filtration by the glomeruli, and consequently the urinary concentration is raised.

The nature of the process by which these substances are recovered from the glomerular filtrate has not yet been determined. It has been shown, however, that the rate varies somewhat for different acids (Doty and Eaton, 1941; Pitts, 1943, 1944; Ferguson, Byer and Eaton, 1945). Furthermore, it has been demonstrated (Pitts, 1943) that a maximal rate exists for at least one of them (glycine).

The experiments to be reported in this paper deal with the reabsorption of three monoamino-monocarboxylic amino acids—valine, leucine, and isoleucine—by the dog kidney. They were undertaken in the hope that the results may aid in the ultimate elucidation of the fundamental nature of the process involved.

EXPERIMENTAL PROCEDURE. The experiments were carried out on 5 healthy female dogs which had been trained to submit to experimental manipulation while lying under loose restraint upon an operating table. Urine was collected from the bladder with the aid of an indwelling urethral catheter. Blood samples were collected from the jugular vein in early experiments, but later from the femoral artery.⁴ Collections from the latter were made by the use of an indwelling spinal needle having a tightly fitting stylet and cut to a convenient length. This arrangement proved much more satisfactory than the earlier method of making periodic vena punctures.

After a fasting period of 22 to 24 hours, 50 cc. of water per kgm. of body weight was administered by stomach tube. This served to hydrate the dog and promote the flow of urine. An hour later the bladder was catheterized and washed

¹ Aided by a grant from the Rockefeller Foundation.

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⁴ Although a statistical analysis has not been made, we have never found any appreciable difference between the alpha-amino nitrogen content of arterial and venous blood withdrawn simultaneously. (See also Goettsch et al., 1944.)

with water. Urine was then collected during the next half-hour which constituted the control period. Immediately following this period a blood sample was taken in order that the normal amino acid level of the plasma might be determined. A priming dose, consisting of 100 cc. of an appropriate neutralized solution of amino acid and inulin in physiological saline, was then given intravenously to cause rapid elevation of the plasma concentration. Following this the constant infusion of the solution into the femoral vein was begun and maintained at a rate of about 5 cc. per minute. After 20 to 30 minutes, during which the plasma concentration became stabilized at an elevated level, urine collection was begun. Periods were of 30 minutes' duration, with blood samples of 10 cc. taken at the beginning, mid-point, and end of each. The average amino acid and inulin concentration of these three samples was computed to determine the level over the 30 minute period.

Chemical methods. The amino acid concentration of plasma was determined gasometrically upon picric acid filtrates by the ninhydrin-carbon dioxide method of Hamilton and Van Slyke (1943). Urinary amino acid was likewise determined by the ninhydrin-carbon dioxide method of Van Slyke, MacFayden and Hamilton (1943) after preliminary removal of urea by Squibb's Double Strength urease. All manometric determinations were made in duplicate and were repeated when the values failed to check satisfactorily. Inulin determinations were made upon diluted urine and diluted plasma filtrate according to the colorimetric method of Hubbard and Loomis (1942).⁵ The color intensity was measured with a Coleman spectrophotometer, with a maximum transmission of light at 500 m μ and using a 5 m μ slit. Very accurate values were obtained.

RESULTS. The significant features of the results obtained in these experiments are shown in tables 1, 2 and 3. In accordance with accepted practice, inulin clearance has been used as a measure of glomerular filtration rate (Smith, 1937). The amount of alpha-amino nitrogen filtered per minute has been calculated as the product of the plasma concentration in milligrams per cubic centimeter and the filtration rate in cubic centimeters/minute. This product has been divided by the surface area⁶ of the dog, so that it represents the rate of filtration per square meter of body surface. The amount excreted has been calculated as the product of the urine concentration in milligrams per cubic centimeter and the rate of urine flow in cubic centimeters/minute and likewise represents milligrams excreted per minute per square meter of body surface. The difference between the amount filtered and the amount excreted represents the amount reabsorbed and is recorded in column 9 of each table.

Table 1 shows the results obtained with various plasma concentrations of dl-valine. No attempt was made to determine the amount of alpha-amino

⁵ This method was modified to the slight extent of heating the color-forming solution at 80° C for 14 minutes instead of 8 as prescribed by the authors. This modification assured the maximal development and stability of color, a result which was not always obtained with the shorter period of heating.

⁶ Calculated by the formula of Meeh, where surface area in square meters = $k \sqrt[3]{(\text{body wt. in kgm.})^2}$. For the dog, Rubner has ascribed a value of 0.112 to the constant, k (Lusk, 1928, pp. 122-123.)

nitrogen filtered and reabsorbed during the control period preceding the administration of the acid. Reference to column 8 indicates, however, that the rate of excretion is extremely low in these periods during which the plasma amino acid concentration is at its normal level. Assuming a fairly normal filtration rate,

TABLE 1

The relationship between the amount of valine alpha-amino nitrogen filtered and the amounts excreted and reabsorbed in normal dogs

(1) DOG	(2) PERIOD	(3) URINE FLOW	(4) GLOMERU- LAR FILTRATION RATE	ALPHA-AMINO NITROGEN						(10) Ratio Reabsorbed Filtered
				(5) Plasma conc.	(6) Urine conc.	(7) Filtered	(8) Ex- creted	(9) Reab- sorbed		
				mgm. %	mgm. %	mgm./ min./ sq. m.	mgm./ min./ sq. m.	mgm./ min./ sq. m.		
IV 9.5 kgm. S.A., 0.502 sq. m. (0.5% valine, 0.5% inulin)	Control 1 2	2.50 2.97 3.57	 38.3 43.8	 2.80 8.16 9.86	 0.54 33.3 38.8	 6.24 8.60	 0.027 1.97 2.76	 4.27 5.84	 0.684 0.678	
II 8.65 kgm. S.A., 0.472 sq. m. (1% valine, 0.5% inulin)	Control 1 2 3 4	3.70 4.30 3.30 6.13 8.88	 32.0 44.6 51.8 50.6	 3.46 10.2 13.0 17.1 19.5	 0.42 25.8 57.3 47.2 51.2	 6.90 12.3 18.8 20.9	 0.033 2.35 4.90 6.13 9.64	 4.55 8.30 12.7 11.3	 0.660 0.675 0.675 0.540	
III 9.55 kgm. S.A., 0.504 sq. m. (1% valine, 0.5% inulin)	Control 1 2 3 4	0.50 8.83 11.9 12.1 11.1	 68.8 69.4 70.7 63.0	 4.05 11.6 14.9 17.2 18.2	 4.40 28.4 27.9 34.3 38.7	 15.8 20.5 24.2 22.8	 0.044 4.98 6.59 8.25 8.53	 10.8 13.9 16.0 14.3	 0.684 0.678 0.661 0.627	
II 8.65 kgm. S.A., 0.472 sq. m. (2% valine, 0.5% inulin)	Control 1 2 3 4	2.67 5.00 5.20 4.44 6.80	 49.5 50.1 50.7 46.8	 2.82 20.8 22.4 24.4 29.0	 0.68 81.6 108.9 133.8 128.3	 21.8 23.8 26.2 28.8	 0.038 8.65 12.0 12.6 18.5	 13.2 11.8 13.6 10.3	 0.605 0.495 0.519 0.358	
IV 9.3 kgm. S.A., 0.495 sq. m. (2% valine, 0.5% inulin)	Control 1 2 3 4	2.90 4.43 5.37 7.73 5.46	 40.7 47.5 46.3 43.8	 2.04 24.0 26.7 29.2 30.0	 0.38 91.7 108.4 94.4 125.7	 19.7 25.6 27.3 26.6	 0.022 8.20 11.8 14.8 13.9	 11.5 13.8 12.5 12.7	 0.584 0.539 0.458 0.477	

which seems likely in most cases, it is apparent that reabsorption of amino nitrogen is nearly complete with the plasma concentration at its usual level. In fact Pitts (1943) has presented some limited data to show that under these conditions there is reabsorption of approximately 98 per cent of the filtered alpha-amino nitrogen.

It will be noted in the table that a low rate of urine flow (0.5 cc./min.) is accompanied by a high concentration of urinary amino nitrogen (4.40 mgm. per

TABLE 2

The relationship between the amount of leucine alpha-amino nitrogen filtered and the amounts excreted and reabsorbed in normal dogs

(1) DOG	(2) PERIOD	(3) URINE FLOW	(4) GLOMERU- LAR FILTRATION RATE	ALPHA-AMINO NITROGEN						(10) Ratio Reabsorbed Filtered
				(5) Plasma conc.	(6) Urine conc.	(7) Filtered	(8) Ex- creted	(9) Reab- sorbed		
		cc./min.	cc./min.	mgm. %	mgm. %	mgm./ min./ sq.m.	mgm./ min./ sq.m.	mgm./ min./ sq.m.		
V	Control	0.27		2.88	6.71		0.037			
9.1 kgm.	1	3.27	50.2	4.09	0.80	4.20	0.054	4.15	0.989	
S.A., 0.488 sq. m.	2	5.50	52.3	4.44	0.38	4.75	0.043	4.71	0.992	
(0.5% leucine, 1% inulin)	3	4.23	49.3	4.53	0.50	4.58	0.043	4.54	0.991	
IV	Control	1.17		3.54	1.28		0.031			
8.8 kgm.	1	3.30	41.2	10.0	7.69	8.64	0.531	8.11	0.940	
S.A., 0.477 sq. m.	2	4.70	37.0	9.19	1.77	7.13	0.174	6.96	0.976	
(1% leucine, 0.5% inulin)	3	4.60	47.1	9.83	13.3	9.71	1.28	8.43	0.868	
	4	2.33	40.0	8.84	2.97	7.41	0.145	7.27	0.981	
III	Control	0.63		4.59	4.16		0.051			
9.8 kgm.	1	6.07	52.8	12.6	24.4	13.0	2.89	10.1	0.778	
S.A., 0.513 sq. m.	2	5.10	44.6	13.0	15.1	11.3	1.50	9.80	0.867	
(2% leucine, 0.5% inulin)	3	5.70	52.6	12.2	11.2	12.5	1.25	11.3	0.904	
V	Control	0.23		3.68	6.69		0.031			
9.3 kgm.	1	4.90	52.8	11.9	41.6	12.7	4.12	8.58	0.675	
S.A., 0.495 sq. m.	2	6.90	54.5	13.8	50.0	15.2	6.97	8.23	0.541	
(2% leucine, 0.5% inulin)	3	5.83	49.1	16.0	65.2	15.9	7.68	8.22	0.516	
III	Control	0.60		3.93	3.57		0.041			
10 kgm.	1	2.73	55.7	12.1	36.9	13.0	1.94	11.1	0.854	
S.A., 0.520 sq. m.	2	6.00	56.9	15.2	48.3	16.6	5.58	11.0	0.662	
(2% leucine, 0.5% inulin)	3	8.07	55.6	17.0	54.1	18.2	8.40	9.80	0.539	
VI	Control	*		4.93	*					
8.4 kgm.	1	8.50	40.7	20.9	62.1	18.4	11.4	7.00	0.380	
S.A., 0.463 sq. m.	2	6.84	35.0	24.6	85.6	18.6	12.6	6.00	0.322	
(2% leucine, 0.5% inulin)										

* Not determined.

cent). This relationship can also be seen in tables 2 and 3 and has been noted in human subjects by Kirk (1937).

A few experiments were performed to determine whether an increase in the amount of water administered prior to the experiment would have resulted in an increased urine flow and lower amino nitrogen concentration during the control period. Dog III, for example, was given 50 cc. of water per kgm. of body weight and one hour later urine collection was begun. The rate of flow over the next 30

TABLE 3

The relationship between the amount of isoleucine alpha-amino nitrogen filtered and the amounts excreted and reabsorbed in normal dogs

(1) DOG	(2) PERIOD	(3) URINE FLOW	(4) GLOMERU- LAR FILTRATION RATE	ALPHA-AMINO NITROGEN					
				(5) Plasma conc.	(6) Urine conc.	(7) Filtered	(8) Ex- creted	(9) Reab- sorbed	(10) Ratio Reabsorbed Filtered
		cc./min.	cc./min.	mgm. %	mgm. %	mgm./ min/ sq.m.	mgm./ min/ sq.m.	mgm./ min/ sq.m.	
II 8.7 kgm. S.A., 0.474 sq. m. (0.5% isoleu- cine, 0.5% in- ulin)	Control	0.73		3.85	2.18		0.034		
	1	1.73	32.6	7.19	36.3	4.95	1.33	3.62	0.731
	2	5.37	42.5	8.50	19.8	7.63	2.24	5.39	0.706
	3	5.93	43.0	9.66	22.1	8.77	2.76	6.01	0.685
IV 9.8 kgm. S.A., 0.513 sq. m. (1% isoleucine, 0.5% inulin)	Control	0.93		3.56	1.47		0.027		
	1	6.17	43.1	12.1	26.7	10.2	3.22	6.98	0.684
	2	7.20	49.0	15.4	37.2	14.7	5.23	9.47	0.644
	3	5.93	46.7	18.5	54.7	16.8	6.33	10.5	0.625
IV 11.6 kgm. S.A., 0.574 sq. m. (2% isoleucine, 0.5% inulin)	Control	1.07		3.83	1.31		0.024		
	1	4.87	46.4	14.4	45.3	11.6	3.85	7.75	0.668
	2	7.60	52.7	20.4	67.2	18.8	8.91	9.89	0.526
	3	6.69	47.7	23.7	91.8	19.7	10.7	9.00	0.456
V 10.0 kgm. S.A., 0.520 sq. m. (2% isoleucine, 0.5% inulin)	Control	*		3.63					
	1	4.50	64.5	16.4	109.1	20.4	9.45	11.0	0.539
	2	5.17	54.9	18.5	103.8	19.5	10.3	9.20	0.471
	3	5.80	56.4	20.4	113.7	22.1	12.7	9.40	0.425

* No appreciable urine flow.

minute period was 0.133 cc. per minute and the amino nitrogen concentration 5.82 mgm. per cent. Another 50 cc. per kgm. was then administered and urine collection begun 45 minutes later. The urine flow this time was 3.03 cc. per minute and the amino nitrogen concentration 0.462 mgm. per cent.

In spite of the high concentration accompanying low rates of urine flow, the actual excretion of amino nitrogen per minute is not elevated. This can be seen from the tables and was apparent in the experiment mentioned above. In the

latter, the excretion of amino nitrogen in milligrams per minute per square meter amounted to 0.017 during the first period, 0.031 during the second.

It is seen that as the plasma concentration of valine amino nitrogen is raised, the amount filtered per minute is increased. As the amount filtered increases to about 19 mgm. per minute per square meter, the amount reabsorbed also increases. The amount reabsorbed does not increase quite proportionately to the amount filtered, however, as seen by the decrease in the ratio of amino nitrogen reabsorbed to filtered in column 10. When the amount filtered reaches and exceeds a value of about 19 mgm. per minute per square meter the amount reabsorbed fails to show any consistent increase. An increase is seen in the case of dog III, but there is none in dogs II and IV (lower part of the table). In these two dogs it appears that a maximal rate of reabsorption has been reached at a level slightly below 14 mgm. per minute per square meter. In dog III the level may be somewhat higher. Pitts (1943) has noted that the maximal rate of reabsorption of glycine alpha-amino nitrogen likewise differs somewhat for different dogs.

Table 2 shows the essential data obtained from similar studies upon l-leucine. It is seen that as the amount of alpha-amino nitrogen filtered increased to about 11 mgm. per minute per square meter the amount reabsorbed increased in a regular manner. Above this value, however, there was no consistent increase in reabsorption with filtration. As was the case with valine, the maximal rate of reabsorption of leucine appears to differ somewhat for different dogs. In dog III it lies between 10 and 11 mgm. per minute per square meter and in dog V, between 8 and 9. Dog VI (bottom of table) apparently has a somewhat lower limit of between 6 and 7 mgm. per minute per square meter. Unfortunately, the data on this dog are limited to this single experiment, as she had to be discarded subsequently because of difficulties involved in training her to lie quietly on the table.

Another interesting fact shown in this table is the efficiency of the reabsorptive process when the plasma amino nitrogen concentration is raised to levels up to 2.5 times the normal (dog V and dog IV, periods 2 and 4, upper part of table). Under these conditions, reabsorption was found to be from 98 to 99 per cent complete.

The results obtained in experiments with dl-isoleucine were essentially similar to those with dl-valine and l-leucine, as can be seen from table 3. There is a progressive increase in the rate of reabsorption as the amount filtered increases up to about 17 mgm. per minute per square meter. As in the case of the other two acids, however, the proportion of filtered amino nitrogen which is reabsorbed falls off somewhat, and consequently the fraction excreted increases. Increases above 17 mgm. per minute per square meter in the amount filtered are not accompanied by a further rise in the reabsorption rate which levels off between 9 and 11 mgm. per minute per square meter.

Figure 1 shows graphically the relation of the amount of alpha-amino nitrogen reabsorbed to that filtered for all of the experiments performed with the three amino acids. In each case, the curve has been leveled off to indicate the average

rate of reabsorption beyond the last of the points for which an increase in the amount filtered was consistently accompanied by an increase in the amount reabsorbed.

From the data plotted in this figure it is apparent that at all lower levels of filtration the rate of reabsorption of leucine alpha-amino nitrogen is somewhat higher than that of valine and isoleucine. With all three, the maximal rate is approached gradually as it is with glycine, alanine, glutamic acid and arginine (Pitts, 1943, 1944). Since reabsorption fails to increase in direct proportion with filtration, there is of course a continuous increase in the amount of amino acid excreted prior to the attainment of a maximal rate. In this respect, the

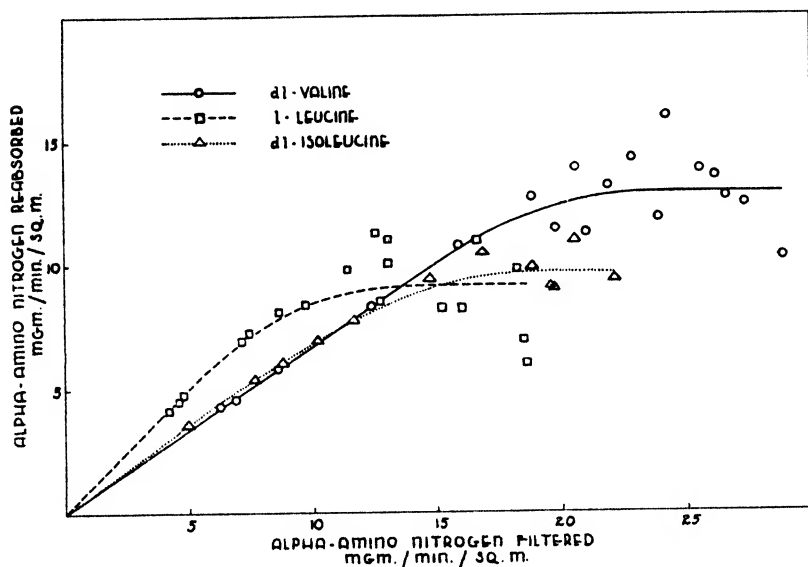


Fig. 1. The relation between renal filtration and reabsorption of alpha-amino nitrogen of dl-valine, l-leucine, and dl-isoleucine in dogs.

treatment of amino acids by the kidney differs from that of glucose. In the case of the latter, the reabsorptive process keeps pace with filtration until the maximal rate is reached, and only then are significant amounts excreted in the urine (Shannon and Fisher, 1938).

The maximal levels attained by leucine and isoleucine are approximately the same, the average value for each lying between 9 and 10 mgm. per minute per square meter. There is little doubt that the level for valine is somewhat higher, averaging about 13 mgm. per minute per square meter. A possible interpretation of these findings is discussed below.

DISCUSSION. Pitts (1943, 1944) has postulated a general mechanism for the renal reabsorption of amino acids which is based upon a hypothesis developed by Shannon and Fisher (1938) in connection with glucose reabsorption. It is

suggested that the amino acid, A, in the tubular lumen combines with a stable component, B, present in constant but limited quantity in the tubule cells. Decomposition of this complex, AB, delivers A to the peritubular interstitial fluid. It is assumed that the rate of decomposition of AB is high in relation to the rate at which equilibrium is attained in the reaction by which A and B combine. While A is present in the tubules in amounts insufficient to saturate B, the rate of reabsorption is limited by the concentration of A and the velocity of its combination with B. Consequently, there will be a gradual increase in the rate of reabsorption as the concentration of A increases. The lower the velocity of combination, the more gradually B will become saturated. When the concentration of A becomes sufficient to saturate B completely the rate of reabsorption becomes constant and independent of the concentration of A. It is then limited by the velocity of decomposition of AB, assuming the total amount of B to be fixed.

We have no information regarding the nature of the hypothetical cellular component, B, nor of the possible reactions by which the complex between it and the amino acid, A, is formed or decomposed. Nevertheless, the above hypothesis is of considerable aid in the interpretation of some of the results obtained in the foregoing experiments. Reference to figure 1 indicates that the relation of reabsorption to filtration follows the same general pattern for each of the three amino acids tested. We might assume, for the sake of discussion, that they are all reabsorbed by the same mechanism. Although there is no experimental evidence for this assumption, it does not seem unlikely in view of the similarity of the chemical structure of valine, leucine and isoleucine, and in view of the fact that Pitts (1944) has presented evidence that glycine, alanine, glutamic acid, and arginine are reabsorbed by a common mechanism.

It will be noted that the maximal reabsorption rate attained by valine is somewhat higher than the levels attained by leucine and isoleucine. Furthermore, the attainment of the maximal level for valine requires a considerably higher concentration of tubular alpha-amino nitrogen. In the light of Pitts' hypothesis, these facts might be interpreted as indicating that larger amounts of valine are required to saturate the cellular component B and thus make the rate of decomposition of the AB complex the limiting factor of the reabsorptive process. With such an interpretation, it is unnecessary to postulate a higher rate of decomposition of the AB complex as being necessary for the maintenance of a higher maximal rate of reabsorption for valine, since unit for unit, B is capable of transferring a greater number of molecules of valine than of leucine or isoleucine. Thus, the amount of B available for valine is *in effect* greater than that available for leucine and isoleucine. Whether the shorter chain-length of valine is in any way related to this higher maximal rate is not known.

The similarity in slope of the curves for valine and isoleucine prior to the leveling off of the latter as the maximal rate is approached indicates a similarity in the rate of combination of these two acids with the B component. From the greater slope of its curve, it appears that leucine combines somewhat more rapidly. Consequently, it approaches the maximal rate more abruptly than do the

other two substances. No reason can be suggested to explain why leucine should combine more rapidly with the B component. In view of the findings of Pitts (1944) that at presaturation levels the rates of reabsorption of glycine, alanine, glutamic acid, and arginine are inversely related to the length of their carbon chains, it might be expected that the valine curve would manifest the greatest slope of the three amino acids tested.

SUMMARY

1. The renal reabsorption of alpha-amino nitrogen of dl-valine, l-leucine, and dl-isoleucine has been studied at various plasma concentrations obtained by infusion of the individual amino acids.

2. In each case, the rate of reabsorption increases with the amount filtered and gradually approaches a maximal limit. At lower levels of filtration, the rates of reabsorption of valine and isoleucine are approximately the same and are somewhat lower than that of leucine. At higher levels of filtration, the maximal limits of reabsorption attained by leucine and isoleucine are of about the same magnitude whereas that of valine is somewhat higher.

3. These results are discussed in terms of a recently proposed general hypothesis for the renal reabsorption of amino acids.

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SUPPLEMENTARY EFFECTS OF ARSENIC AND MANGANESE ON COPPER IN THE SYNTHESIS OF HEMOGLOBIN¹

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The medical practice of incorporating small amounts of inorganic arsenic into tonics for man and beast apparently has some merit because of its continued use for many years. However, results of most of the investigations planned to ascertain the rôle of arsenic in the economy of animals have had a negative trend (1-6). In their report on the inability of other trace elements to replace copper in hematopoiesis Waddell and co-workers (2) state "The effect of arsenic is minimal and temporary." Although concluding that the daily requirement, if any, of the rat for arsenic is not more than that contained in 50 ml. of milk (about 2 micrograms), Hove *et al.* (5) observed that arsenic supplementation delayed the fall in hemoglobin level associated with removal of rats from a diet of whole milk supplemented with iron, copper and manganese to one of whole milk.

The occasional reports of beneficial effects of arsenic, even though small, made the subject of sufficient importance to justify further investigation. Therefore experiments were planned to ascertain if arsenic has an essential rôle in the economy of animals. In addition to observing a beneficial effect of arsenic upon hemoglobin, a similar effect of manganese was noted so regularly in these experiments that the responses to both elements are reported in this paper.

Use of dry ration. The basal ration used in the first part of the investigation was a milk powder-sucrose ration of the following percentage composition: vitaminized casein,² 1.3; skim-milk powder, 50.0; sucrose, 47.7; and fortified corn oil,³ 1.0. Concentrations of the mineral supplements (expressed in p.p.m. of ration) were as follows: iron, 77.7; copper, 7.7; manganese, 7.7; and arsenic, either 1 or 5. The elements were added in the form of ferric citrate, copper sulfate, manganous sulfate and sodium arsenate. All were analytical grade but were not specially purified. The concentrations of iron, copper and manganese used are approximately those resulting (on solids basis) when milk is supplemented with these elements at 1.0, 0.1 and 0.1 mgm., respectively, per 100 ml. of milk.

Young rats were prepared for use in the first experiment by placing stock colony rats with the litters, when the latter were two weeks of age, in cages pro-

¹ The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director.

² Thiamine hydrochloride, 20 mgm.; pyridoxine hydrochloride, 20 mgm.; riboflavin, 50 mgm.; calcium pantothenate, 0.1 gram; nicotinic acid, 5 grams; p-aminobenzoic acid, 5 grams; inositol, 10 grams; choline chloride, 10 grams; casein, 100 grams.

³ Carotene, 0.133 gram; viosterol, 1 gram; alphatocopherol, 0.4 gram; 2-methyl-1,4-naphthoquinone, 10 mgm.; corn oil, 100 grams.

vided with wire false bottoms and feeding them a diet of whole milk until the young weighed 40 to 50 grams. A total of 36 rats, 18 of each sex, were then divided into 3 equal groups which were fed the basal ration, basal plus 1 p.p.m. arsenic, and basal plus 5 p.p.m. of the element, respectively. By way of eliminating variables other than individual differences, distribution of animals among the 3 groups was made from groups of 3 litter mates of the same sex and approximately the same weight. Average weights of animals in the 3 groups initially and at weekly intervals for 9 consecutive weeks are recorded in table 1. It will be noted that with the exception of the first two weeks weights of the control rats equaled or exceeded the weights of animals in the arsenic groups. By the fifth week a retarding effect of arsenic at the higher level of intake became noticeable.

TABLE 1
Growth of rats at different levels of arsenic intake

WEEK NO.	WEEKLY WEIGHTS		
	Controls	1 p.p.m. As	5 p.p.m. As
	<i>grams</i>	<i>grams</i>	<i>grams</i>
(Initial)	43	43	43
1	65	64	69
2	96	94	99
3	125	120	125
4	147	141	147
5	171	167	165
6	186	179	177
7	198	194	189
8	210	205	200
9	219	215	191

That supplementation with arsenic during the 9-weeks feeding period exerted a demonstrable effect upon the concentration of hemoglobin is evident from the data in table 2. It may be seen that of the 12 animals fed 1 p.p.m. of arsenic 8 exceeded and 3 fell below their control mates, the average for the group being 103 per cent of that of the controls. Rats ingesting arsenic at a level of 5 p.p.m. of ration held a slightly better advantage over the controls in that 9 of them had hemoglobin concentrations above and only 2 below their respective control mates. Their hemoglobin levels at the end of the 9-weeks feeding period averaged 108 per cent of the controls.

Following this observation that arsenic increases the concentration of hemoglobin, an experiment was planned to ascertain the length of time required for the effect to be discernible and the influence of manganese and/or copper upon its magnitude. Young rats were prepared for use in the experiment as previously described except that in place of whole milk the unsupplemented basal ration was fed to the mothers and young. Originally it was intended that the rats should be rendered quite anemic before being started on the respective supplements, but the ration contained sufficient copper (2.85 p.p.m.) to prevent this.

Scarcity of sucrose necessitated substitution of glucose (Cerelese) as a source

of carbohydrate during about half of the feeding period. In the absence of skim-milk powder for 2 weeks casein, lactalbumin, lactose, salts and fat were mixed approximately in the proportions occurring in the powder and incorporated into the ration at the same level. Supplements fed to the respective groups were as follows: 1, arsenic; 2, iron; 3, iron and arsenic; 4, iron and copper; 5, iron, copper and arsenic; 6, iron, copper and manganese; and 7, iron, copper, manganese and arsenic. Iron, copper and manganese were fed at the respective levels employed in the preceding experiment and arsenic at the 5 p.p.m. level only. Forty-two young rats were distributed among the 7 groups equally according to sex (3 each per group) and as nearly as feasible according to body weight

TABLE 2
Effect of arsenic supplementation upon level of hemoglobin

MATES NO.	CONCENTRATION OF HEMOGLOBIN			RELATIVE CONCENTRATIONS	
	Controls	1 p.p.m. As	5 p.p.m. As	1 p.p.m./controls	5 p.p.m./controls
	gram/100 ml.	gram/100 ml.	gram/100 ml.		
1	14.7	16.8	17.1	114	116
2	16.0	16.4	18.4	103	115
3	16.0	17.8	15.7	111	98
4	17.0	17.6	18.2	104	107
5	18.1	18.2	17.8	101	98
6	16.2	16.7	19.3	103	119
7	18.4	17.6	18.4	96	100
8	16.0	15.8	17.9	99	112
9	16.3	16.8	17.5	103	107
10	17.4	16.7	18.2	96	103
11	14.3	16.0	17.9	112	125
12	19.3	19.3	19.9	100	103
Avg.....	16.6	17.1	18.0	103	108

and hemoglobin level. The animals were weighed and hemoglobin determinations made at weekly intervals.

In none of the groups did arsenic accelerate the rate of growth; however the concentration of hemoglobin was favorably affected by arsenic supplementation as shown in figure 1. It will be noted upon examination of the curve of the Fe-group that relatively slow but continuous regeneration of hemoglobin occurred, this response doubtless being caused by the copper in the basal ration. Arsenic was without effect when iron constituted the only other supplement. In the groups receiving iron and copper, however, responses to supplementation with arsenic and/or manganese were apparent. For example, the curve for the Fe+Cu-group falls well below that of the Fe+Cu+As-group. Except for the unexplained drop in two instances in the Fe+Cu+As-group, the effects of either or both of these supplements when added to iron and copper were noticeable after the first week. When administered singly, manganese exerted the more powerful effect, together their effects were additive. The hemoglobin con-

centrations for each group during the entire period of 10 weeks yield the following averages: As-group, 13.2; Fe—14.6; Fe + As—14.8; Fe + Cu—15.2; Fe + Cu + As—15.6; Fe + Cu + Mn—16.0; Fe + Cu + Mn + As—16.7 grams per 100 ml.

Use of milk-glucose ration. The basal ration in this series consisted of whole milk to which glucose (Cerelese) was added at the rate of 13 grams per 100 ml. of milk. Hence the general composition of the basal ration was much the same

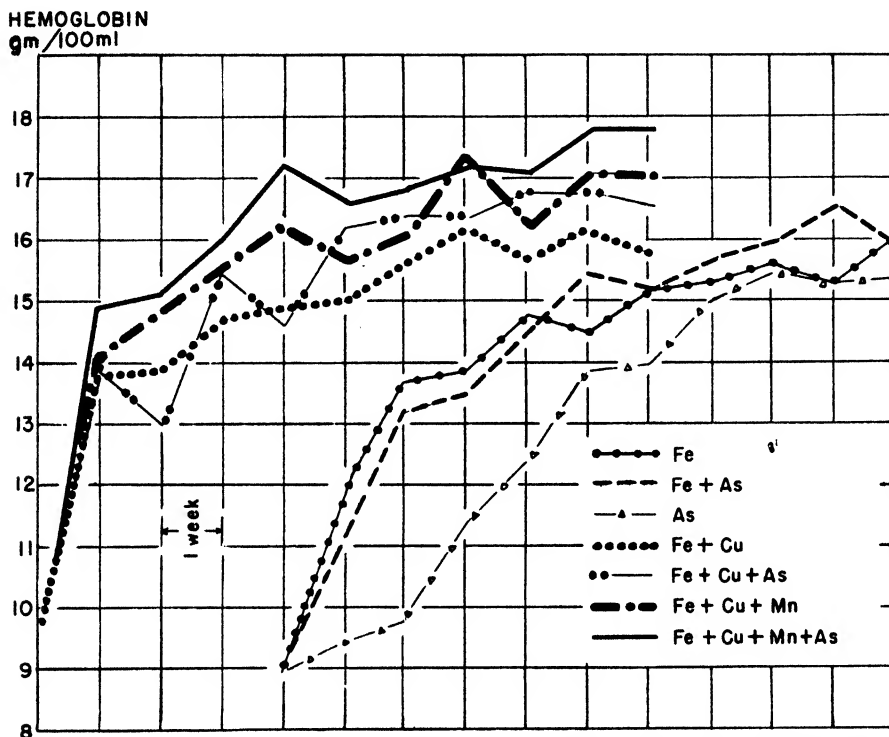


Fig. 1. Effect of arsenic and manganese supplements upon concentration of hemoglobin when added to a skim-milk powder-sucrose ration.

as the dry one previously described. Supplements in this series, as in the one immediately preceding, furnished on solids basis 77.7 p.p.m. iron, 7.7 p.p.m. copper, 7.7 p.p.m. manganese and 5 p.p.m. arsenic.

Young rats were prepared for an experiment by the usual procedure, the ration being the unsupplemented milk-glucose diet. With this diet it was possible to reduce hemoglobin levels to 5 or 6 grams per 100 ml. within about 3 weeks after weaning, at which time the rats were placed on the respective supplements. As far as possible rats from a given litter were distributed among the various groups, but it was considered of prime importance that sexes be equalized (3 each per

group) and that hemoglobin levels, and weights if possible, be approximately equal.

Of the animals fed arsenic as the sole supplement, 3 died within 5 weeks and the others barely survived for the 10-weeks period having hemoglobin values of

HEMOGLOBIN
gm/100ml

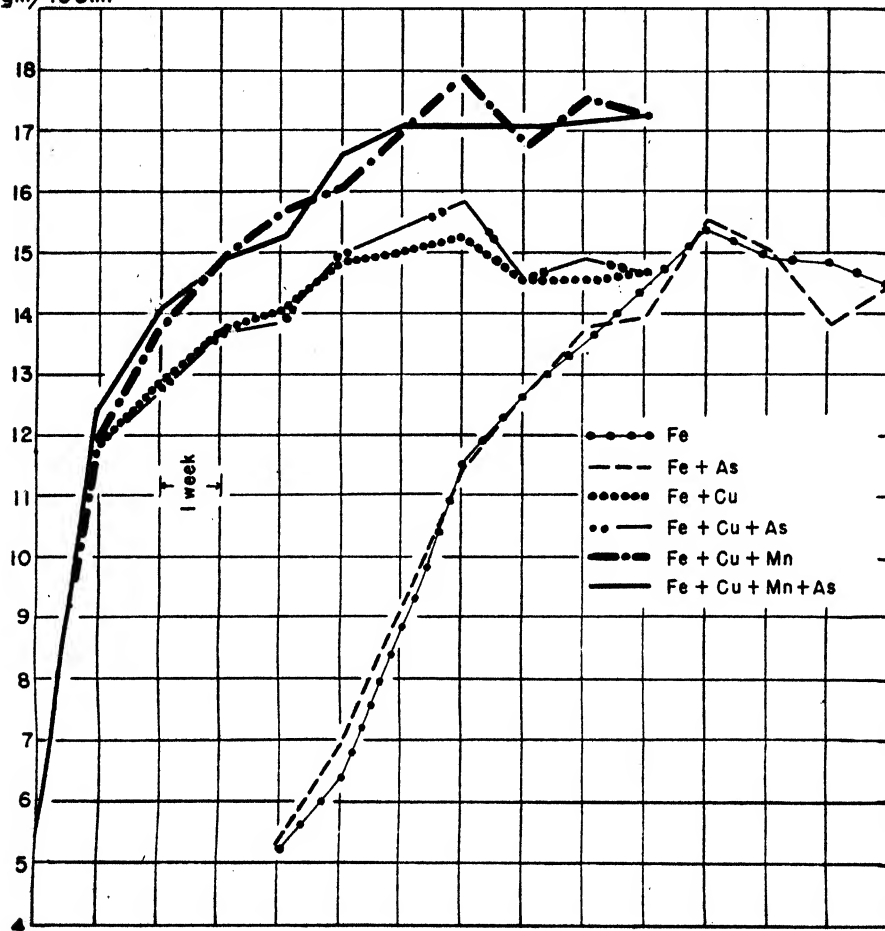


Fig. 2. Effect of arsenic and manganese supplements upon concentration of hemoglobin when added to a milk-glucose ration.

approximately 3 grams per 100 ml. near the close of the period. With regard to growth, arsenic exerted no effect whatsoever whereas manganese-supplemented animals grew faster, as reported by other investigators, than those limited to supplements of iron and copper. The average concentrations of hemoglobin

for each of the 6 groups surviving the feeding period are recorded in figure 2. It is to be noted that sufficient copper was contained in the rations of the Fe- and the Fe + As-groups for continuous, though somewhat retarded, hemoglobin synthesis, so that at the conclusion of the experiment these groups had approximately the same level of hemoglobin as those that received the Fe + Cu-supplement. The effect of manganese upon the hemoglobin level was even more pronounced than that observed in the previous experiment. It was quite noticeable after the first week and became more pronounced as the experiment progressed. Average hemoglobin values for each of the groups during the 10-weeks period were as follows: Fe-group, 12.7; Fe + As—12.7; Fe + Cu—14.2; Fe + Cu + As—14.3; Fe + Cu + Mn—15.9; Fe + Cu + Mn + As—15.9. From these data it will be observed that the two groups receiving the manganese supplement averaged slightly greater than 12 per cent more hemoglobin throughout the period than did animals fed iron and copper supplements only.

DISCUSSION. Why animals on the dry ration should have responded to arsenic supplementation whereas those on the milk-glucose ration did not is unknown. Certainly the composition, so far as solids were concerned, did not differ appreciably except perhaps for impurities acquired by the skim-milk powder during its manufacture. Since the effect of arsenic, observed when rats were fed the skim-milk powder-sucrose ration, did not diminish during relatively long feeding periods, its action could not be termed transitory as was characteristic of the response noted by Waddell *et al.* (2). In connection with the positive effect of arsenic reported herein, it is of interest to note that Guthmann and Grass (7) believe that arsenic has a direct relationship to growing tissue and cell proliferation. Increased length of life of red blood cells through greater resistance to hemolysis, was postulated by Hove and co-workers (5) as the manner by which arsenic delayed the fall in hemoglobin which they noted when iron, copper and manganese supplements were withheld from rats on a milk diet. No attempt was made in the present investigation to ascertain the manner by which arsenic raised the level of hemoglobin.

Manganese has been reported previously to play a part in synthesis of hemoglobin (8-10). Whereas these investigators found manganese to be almost as effective as copper, the effect noted by us was that of supplementing the action of this element. Mitchell and Miller (11) observed a response to manganese but reported that they considered the effect negligible. To be sure, our results do not contraindicate a unique rôle of copper in hematopoiesis. Since copper was fed at a level of 7.7 p.p.m. solids (equivalent to adding 0.1 mgm. of copper to 100 ml. of milk), there was no deficiency of this element and hence the increased level of hemoglobin could not have been due to traces of copper in the manganous sulfate.

CONCLUSIONS

Rats responded to arsenic supplements with increased levels of hemoglobin when fed a basal ration composed mainly of skim-milk powder and sucrose (or glucose) and adequately supplemented with iron and copper.

Manganese supplementation of the skim-milk powder-sucrose or whole milk-glucose ration, to which adequate iron and copper were added, resulted in increased synthesis of hemoglobin and enabled rats to maintain a higher level than when the basal rations were fortified with iron and copper only.

With rats fed the dry ration the combined effect of manganese and arsenic upon the level of hemoglobin was greater than that of either supplement alone.

Under conditions of these experiments arsenic supplementation did not affect rate of body growth.

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THE DEVELOPMENT AND CONTOUR OF CARDIAC INJURY POTENTIAL¹

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It has long been known that in leads from an uninjured and an injured region of heart muscle, potential time curves may be obtained when the heart becomes active which are purely monophasic and represent a rapidly developing difference in potential between the electrodes, with a much slower but continuous return to the resting state. In many instances, however, the curves obtained show departures from this simple type, with one or more initial peaks or interruptions in the initial rapid potential change and with modifications in the smooth continuous return to the resting state. Injuries of various kinds have been used by different investigators, cuts, local burning or crushing, application of various chemicals, local pressure and by exposing a local region to suction. With the exception of the last two methods of injury rarely have uncomplicated monophasic curves been obtained. Jochim, Katz and Mayne (1) obtained satisfactory monophasic curves from the mammalian heart by local pressure. The use of local suction to produce injury was first employed by Schutz (2). He applied suction by means of a small tube and vacuum pump and tied off the tab of tissue sucked into the tube at its base. The tube was then removed and an electrode applied to the tab. H. C. Wiggers (3) modified this procedure by allowing the tube to remain with continuation of the suction and by making contact with the tab by means of an electrode within the suction tube. The tab was not tied off. This method of injury in our experience produces the most consistent and least complicated type of injury potential curve. By the application of suction of increasing increments the development of the monophasic injury potentials may be studied and curves recorded representing transitions from the action potentials recorded before suction to the complete injury potential. In many cases following the release of suction similar transition curves with the final return to normal or near normal action potentials may be obtained.

In the bipolar type of injury potential from the heart, that derived from two leads from the heart, one from an uninjured region and one from an injured region, there has been until recently general acceptance of the view that the potential change associated with activity occurs entirely at the uninjured region. According to this view, the resting negative potential at the injured region remains unchanged when the remaining muscle is active and the potential recorded represents the true action potential of normal cardiac muscle. This interpretation accords with the classical theory of "negativity," according to

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which the active tissue develops a negative potential which declines as the muscle returns to the resting state. It is impossible however to determine whether a potential difference developing between the two electrodes of a bipolar lead results from a change of potential of one sign under one of the electrodes or a change of opposite sign under the other electrode. By the use of unipolar leads, one electrode on an injured region of the heart and the other from a region of the animal distant from the heart, Wilson and co-workers (4) showed in 1933 that there was a disappearance of the negative potential of the injured region when the surrounding regions become active and Eyster, Meek, Goldberg and Gilson (5) later showed that the injured region, negative with respect to uninjured resting muscle, became positive with respect to the same reference during contraction. These observations have been confirmed by several groups of workers (6)(7)(8). Other recent workers, however, (9)(10) fail to accept this evidence and maintain the view that the potential changes in injury are derived wholly from uninjured regions of cardiac muscle.

The present work is concerned with the development of activity injury potentials with especial reference to the complications in wave form that may occur and the seat of origin of the potential changes.

METHODS. Most of the experiments were carried out on the hearts in situ of large specimens of snapping turtle (*Chelydra serpentina*) under nembutal anesthesia and artificial respiration. In a few instances the hearts were isolated and in others cardiac strips from the sinus or sino-auricular regions were employed. Localized injuries were produced in most instances by the suction method of H. C. Wiggers, the suction tube employed having an internal diameter of 1 mm. The injury potential time curves were recorded by means of a direct coupled amplifier and one element of a three channel cathode ray oscillograph. In all but a few instances a reference curve was recorded by means of a second amplifier of the same design in a second channel of the cathode ray tube. In several experiments three potential time curves were recorded simultaneously, using all three channels of the cathode ray tube and three identical direct coupled amplifiers.

The suction tube contains a zinc-zinc sulphate electrode with the wick making contact with the heart surface enclosed by the suction tube and constitutes one lead to the input of the amplifier. The second lead completing the circuit is from a second zinc-zinc sulphate wick electrode. The position of this second electrode determines the type of potential time curve which is obtained. If placed on the heart at a considerable distance from the suction electrode, the curve obtained is designated as a bipolar curve. If placed on a region of the animal distant from the heart, (or in the case of an isolated heart surrounded by a conduction medium, it is placed in this medium at a considerable distance from the heart), the curve obtained is a unipolar curve. If placed on the heart surface immediately contiguous with the outside of the suction electrode, and thus only a few millimeters or less from the electrode inside the suction tube, a differential potential time curve is obtained. Finally, if the outside of the suction tube is surrounded by a cloth jacket saturated with saline solution and the wick of the

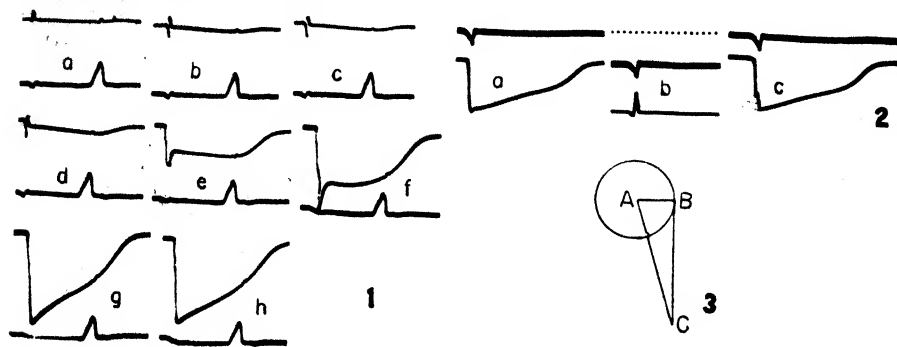
second electrode brought in contact with the cloth jacket, a coaxial curve results. It is to be noted that one may obtain normal action potentials by these various leads before suction is applied to the suction electrode as well as injury potentials after the application of suction.

The potential time curves were recorded on standard bromide paper 6 cm. in width driven at known speeds; in most records at the rate of 25 mm. per second. The sensitivity of the recording systems was determined in all cases by calibrators built into the amplifiers. Examples of calibration are shown at the end of records *b* and *d* of figure 4. Measurements of time intervals and potentials were made by means of a curve analyzer equipped with vertical and horizontal micrometers and a microscope magnifying 32 diameters (11). Measurements of potentials are given in millivolts (mv).

The potential reference in all cases is the potential of the uninjured heart muscle during diastole. Points on curves above this level are recorded as negative, below this, as positive potentials. Since our amplifiers are free from appreciable drift at the sensitivities employed in this work, these levels of reference potentials may be used in successive records. Thus in the description of injury potential curves the potentials stated are measured from the reference level derived from curves before the injury was produced.

The development of the injury activity potential with increasing injury. The use of the suction electrode makes possible recording of the potential developed at a region before injury and at the same region with varying degrees of injury produced by varying amounts of suction or negative pressure. Figure 1 illustrates an experiment of this type. In each of the records the upper tracing is the potential curve from a differential suction electrode on the mid region of the right atrium. The lower tracing is a reference curve from the great vessels and the leg. In record *a* there was no suction and a normal differential curve from the auricle is recorded. There is also evident a considerably smaller component due to ventricular activity. Record *b* was recorded $\frac{1}{2}$ minute after *a* when the region under the suction electrode was exposed to a pressure 25 mm./Hg below atmospheric pressure. There is evident some change in the potential at the onset of auricular activity and a small depression of the curve throughout the period of auricular activity. This is due to the beginning development of a resting injury potential negative with respect to uninjured resting muscle and an activity injury potential positive with respect to the same reference. Record *c*, made $\frac{1}{2}$ minute later and under a negative pressure of 50 mm./Hg shows a slight increase in this depression. Record *d* made $1\frac{1}{2}$ minutes later and under a negative pressure of 75 mm./Hg shows a potential before auricular activity of -2.2 mv. reaching $+4.4$ mv. during auricular activity. Record *e* made $\frac{1}{2}$ minute later at a negative pressure of 100 mm./Hg shows a resting injury potential of -8.5 mv. At the onset of auricular activity this potential rapidly changes to $+14.2$ mv. which declines after a short interval to $+6.0$ mv. and then increases to $+7.1$ mv. before returning to the resting level at the end of auricular activity. There is evidence of some effect of the original differential action potential at the peak of the initial downward movement. Record *f* made one minute later and under a negative

pressure of 125 mm./Hg is similar except that all voltages are increased and there is no trace of the original action potential in the curve. The potential at the onset of auricular activity is -12.8 mv. which rapidly changes to $+27.0$ mv. with the onset of activity, is then reduced and remains at approximately $+14.2$ mv. until it finally declines to the original negative potential of -12.8 mv. at the end of auricular activity. In record *g*, taken 1 minute later and with a negative pressure of 150 mm./Hg, the voltage at the start is -14.2 mv. and at the peak $+29.0$ mv. All trace of original action potential has disappeared. There remains a slight upward convexity following the initial positive peak. Record *h*, taken 1 minute later at a negative pressure of 175 mm./Hg shows an uncomplicated



Figs. 1-3

Fig. 1. Stages in the development of an injury potential from the right auricle of the turtle, produced by suction. For further description see text.

Fig. 2. Comparison of the injury potential curves from bipolar and coaxial leads. Suction injury on ventricle of turtle. The dots at the top of record *b* record time intervals of $1/20$ sec. For further description see text.

Fig. 3. Disposition of leads used in obtaining the records shown in figure 2. The circle represents a suction electrode surrounded by a cloth jacket. *A* represents a wick electrode inside the suction tube. *B* represents a wick electrode connected with the jacket. *C* represents a wick electrode in contact with an uninjured region of the ventricle about 20 mm. distant from the suction electrode.

plicated monophasic curve with the exception of the small ventricular component. The voltages are the same as in the previous curve.

The above description is typical of a considerable number of similar experiments that we have performed. During the development of the typical injury activity potential there appear remains of the original action potential from the region and the occurrence of varying potential changes which cause departures from an uncomplicated smooth monophasic curve. The initial sharp positive peak with rapid decline and maintenance for a period of a lower positive potential, such as are shown in figure 1, records *e*, *f* and *g*, constitute a feature of these curves during their development.

In many instances the changes induced by suction and leading to the production of the injury potential are partially or completely reversible on the release

of the negative pressure. During the recovery from a typical injury potential to a normal or nearly normal action potential transition curves may be obtained similar to those present during the development of the injury potential.

Inclusion of action potentials in injury potential curves. In any method of recording potentials from an injured region there exists the possibility of inclusion of action potentials derived from the electrode on the uninjured region or from regions intervening between the two electrodes. The extent of this inclusion varies with several factors. It is greatest in general when bipolar leads are made from two rather distantly separate regions, one injured and another uninjured, on the same cardiac chamber. Smaller action potential inclusion would be expected in unipolar leads, with one electrode distant from the heart, in differential leads where the second electrode is on uninjured tissue immediately contiguous to the injured tissue and especially in coaxial leads. In the last case potential changes in normal tissue around the injured region would tend to balance out and have little effect on the curve recorded. This is illustrated in figure 2. The upper tracing in each of the records is a reference curve recorded from the great vessel and a hind limb. The lower tracings are records of injury or action potentials recorded as illustrated in the diagram of figure 3. A coaxial suction electrode was placed on the mid region of the ventricle of a large snapping turtle and injury produced by application of a negative pressure of 250 mm./Hg. Record *b* of figure 2 records the normal action potential of the bipolar lead between uninjured tissues at B (surrounding the injury) and at a distant point on the ventricle C. The bipolar action potential is diphasic with initial change in the direction of positive potential and a larger subsequent negative phase. Record *a* records the potential between A and B. The injury potential is uncomplicated by action potential. Record *c* records the potential between A and C. Included in the injury potential are clear evidences of the action potential arising at C and in regions between C and the electrode on the injured region. The first positive phase of the action potential results in a slow rather than abrupt onset of the curve and the negative peak of the action potential is evident as an interruption of the initial positive phase of the injury potential. The total voltage change of the injury potential in *a* of figure 2 of 26.0 mv. is reduced to 24.0 mv. (record *c*) by the inclusion of the negative phase of the action potential.

The extent of the action potential inclusion in an injury potential curve, whatever type of leads are employed, depends also in part on the magnitude and direction of the action potential and the time of its occurrence. Figure 4 illustrates this. Record *a* shows a reference tracing (upper curve) and the action potential recorded by a coaxial electrode from a region on the turtle ventricle. The action potential is diphasic with a negative phase of -12.0 mv. the peak of which is coincident with the peak of the reference curve. Record *b* shows the injury potential tracing obtained following the application of a negative pressure of 260 mm./Hg. It shows inclusion of the action potential with peaks coinciding in time with the peaks of record *a*. Figure 4, *c* and *d* show similar curves obtained from another region on the same ventricle. Record *c* shows the normal action potential. It is monophasic in the negative direction with a maximum

potential of -6.5 mv. and with its peak occurring 0.044 seconds after the peak of the reference curve. In the injury potential from the same region after the application of suction (record *d*) and which starts coincidentally with the reference peak, there is no evidence of action potential remaining. If present it is included in and obliterated by the rapidly developing positive phase of the injury potential.

Comparison of bipolar and unipolar leads from two separate regions before and after injury. Figure 5 records the potentials from an experiment of this type. Two suction electrodes were placed about 15 mm. apart on the anterior surface of the ventricle of a large turtle, at regions designated as A and B. A third electrode was placed on the left hind leg of the turtle (designated as lead I). Connections were made to the recording apparatus so that the potentials recorded would follow Kirchhoff's law such that $AB + BL = AL$. Records *a*, *b* and *c* of

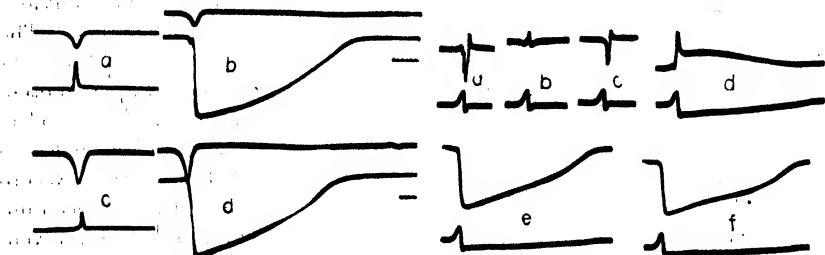


Fig. 4

Fig. 5

Fig. 4. Illustrating the relation of action potentials to injury potentials in injuries from two separate regions on a turtle ventricle. Calibrations of 10 mv. are shown at the ends of records *b* and *d*. For further description see text.

Fig. 5. Comparison of bipolar and unipolar leads from two separate regions on a turtle ventricle, before and after injury. For description see text.

figure 5 were made before injury by suction was produced. The upper curve of *a* records the bipolar action potential AB, while the upper curves of *b* and *c* record the unipolar action potentials from B (BL) and A (AL) respectively. Injury of the regions A and B was now produced by application of negative pressure to each of 250 mm./Hg. The upper traces of records *d*, *e* and *f* are records of the potentials between AB (bipolar curves between two injured regions) and the unipolar curves from injuries at B and A respectively. As in such cases in general, the injury potentials from two regions differ in time of onset and in the peak potential developed. The sharp downward swing (in the direction of positive potential) begins 0.011 second in *e* and 0.022 second in *f* before the reference peak. The maximum voltage change in *e* is 25.5 mv. and in *f* 21.0 mv. If these two injury curves were identical in time of onset, contour and voltage, the potential between them or the potential of curve *d* would be zero, which as is evident is not the case. Curve *e* in addition shows the inclusion to a small extent of action potential at its start, while this is not apparent in curve *f*. This is due to the earlier start of the action potential in tracing *b* than in tracing *c*.

The potential time curves of figure 6 record the results from a somewhat similar experiment but dealing with injured regions on the two auricles. The upper curve records the potential between two suction electrodes on the right and left auricle respectively (RaLa). The middle curve is a record of the potential between a suction electrode on the left auricle and a wick electrode on the ventricle (LaV). The lower curve is a record of potential between the suction electrode on the right auricle and the same wick electrode on the ventricle (RaV). The connections are so arranged that $RaLa + LaV = RaV$, following Kirchhoff's law. The horizontal lines represent the potential of uninjured resting muscle. The sensitivities of the recording channels were kept low in order to prevent the injury curves from exceeding the record boundaries. Calibration of the three channels gave sensitivities of 6.5 mv./mm. for the top and lower records and 5.1 mv./mm. for the middle record. One millimeter horizontal distance on the original record represented 0.04 second.

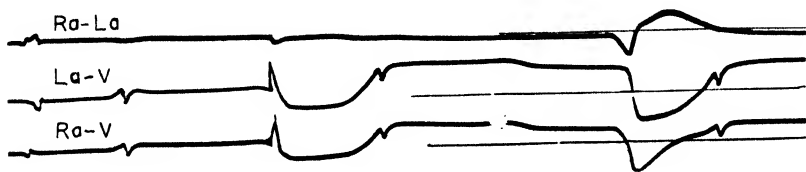


Fig. 6. Simultaneous recordings of injury potentials from the two auricles of a turtle heart. For description, see text.

The first cycle shown records the action potentials before injury was produced. Suction was applied toward the end of this cycle. The injuries are completely developed in the following cycle. The resting injury potentials are $RaLa = +6.5$ mv., $LaV = -27.0$ mv. and $RaV = -20.5$ mv. The maximum positive injury potentials are $RaLa = +34.0$ mv., $LaV = +31.5$ mv. and $RaV = +41.5$ mv. The maximum positive potential of $RaLa$ of $+34.0$ mv. occurs at the time when the voltages of LaV and RaV are respectively -21.0 mv. and $+13.0$ mv. The onset of change in the positive direction with activity occurs simultaneously in $RaLa$ and RaV and 0.1 second later in LaV . Ventricular action potential components appear near the end of the ascending limb of the two auricular injury potential curves. It is evident that the upper curve recording the potentials between the two injuries results from the fact that the two injury curves differ in time of onset, voltages and contour.

The site of origin of the injury potential and the influence of the potentials derived from leads from uninjured tissues. That the injury potential arises primarily from the injured tissue or its boundaries and is dependent on the activity of the cardiac chamber from which it arises is clearly shown by experiments of the type illustrated by figures 7 to 11 inclusive.

Figure 7 (upper trace) records the suction injury potential from the right auricle of a turtle's heart in situ. In record *a* the circuit is completed through an electrode on the pelvis. There is no evidence of inclusion of auricular action

potential components in the curve, but a small ventricular action potential component is evident on the injury curve at the time of ventricular activity as indicated by the reference curve. In *b*, the electrode was moved from the pelvis to the mid ventricle. The first part of the injury potential curve is unaffected. There is however superposed on the ascending limb of this curve a large diphasic ventricular R complex with some distortion of the latter part of the curve due to the ventricular T wave. In record *c*, the electrode was moved from the ventricle

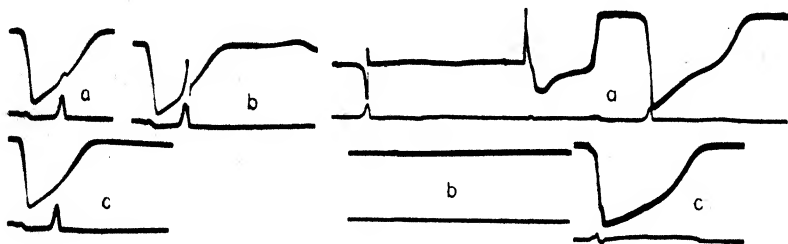


Fig. 7

Fig. 8

Fig. 7. The changes in the injury potential curve of the auricle of a turtle with different positions of the electrode on uninjured tissue. For description see text.

Fig. 8. The effect on a ventricular injury potential of tying a ligature between the auricles and ventricle of a turtle. For further description see text.

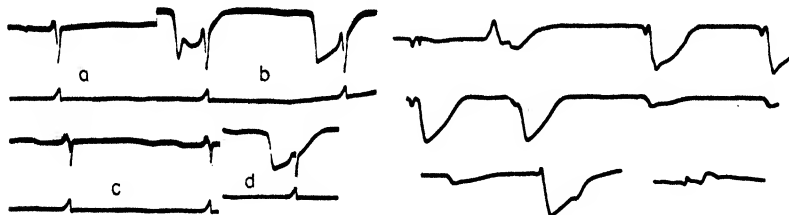


Fig. 9

Fig. 10

Fig. 9. Production of an auricular injury potential and the effects of tying off the injured region. For further description see text.

Fig. 10. Injury potential from a sino-auricular strip. For description see text.

to the auricle on tissue contiguous to the injury, forming a differential injury potential lead. All traces of ventricular components disappear and the auricular injury potential is an uncomplicated monophasic curve.

The records shown in figure 8 were obtained from an experiment in which a suction electrode was placed on the mid ventricle of a turtle's heart in situ. The other electrode was on the left auricle. A reference (lower) curve was recorded by leads from the ventricular apex and pelvis. The first cycle of record *a* shows a small auricular action potential component and a large diphasic ventricular action potential with the initial phase in the positive direction. Following this cycle a negative pressure of 250 mm./Hg was applied to the suction electrode. Within one cycle a complete ventricular injury potential developed with a rest-

electrode on the sinus. The strip was laid on a moist pad. In the first cycle of the top record in figure 10 the separate sinus and auricular action potentials are apparent. Suction with a negative pressure of 200 mm./Hg was applied following this cycle. Within one cycle an auricular injury potential develops with a resting potential of -10.5 mv. followed by a positive potential of $+13.5$ mv. during auricular activity. The action potential of the sinus precedes the injury potential of the auricle. Following the second cycle of the middle tracing a ligature was tied between the injured region and the rest of the preparation. The total voltage change was reduced from 24.0 mv. to 4.0 mv. and consists apparently of the sinus action potential followed by a small injury potential arising from the neighborhood of the ligature. The second cycle in the bottom curve to the left is an injury activity potential resulting from mechanical stimulation of the auricular tissue beyond the ligature. It shows a sinus action potential on its ascending limb. The last curve in this figure was derived from leads from the sinus and from the auricular region on the sinus side of the injury. It shows sinus and auricular action potentials with little or no evidence of injury potentials.

Record *a* in figure 11 is recorded from an automatically beating sinus strip with a suction injury at one end. The total voltage change during the injury activity potential is 25.0 mv. Record *b* was taken after the injured end was ligated off. The total voltage change was reduced to 6.0 mv., which may be regarded as the result of an injury potential arising at the site of the ligature.

The upper tracing in figure 12 is a unipolar injury potential curve derived from the right auricle of a turtle heart *in situ*. The middle tracing is a unipolar injury potential curve from the mid ventricle of the same heart. The lower curve is a reference curve from leads from the great vessels and pelvis. The auricular injury potential starts at the time of auricular activity as indicated on the reference curve. It shows a ventricular action potential component on its ascending limb. The ventricular injury potential starts with the onset of ventricular activity. The durations of the two curves are 1.08 seconds and 1.48 seconds respectively for the auricle and ventricle.

Injury potential from burned or crushed areas. Although we have recorded injury potentials from burned or crushed areas on the turtle heart in numerous experiments we have never obtained the uniform uncomplicated monophasic curve that characteristically occurs after complete suction injury. The curves obtained usually resemble the curves recorded during the development of a suction injury of types shown in figure 1 or during the subsidence of injury following release of suction. In figure 13a the lower trace is the unipolar action potential recorded from the mid ventricle of a snapping turtle. The upper trace in this and subsequent records in this figure is a reference curve from the ventricular apex and pelvis. Record *b* was recorded in a similar manner but with somewhat higher amplification one minute after the region was burned with a hot iron. The injury resting potential is -6.5 mv. while the maximum peak potential during activity is $+4.5$ mv. At least five phases may be recognized in this curve; 1, incorporation of the first part of the action potential to cause the small deflections before the rapid downward movement; 2, the rapid develop-

ing injury potential of -32.0 mv. changing during activity to a positive potential of $+33.0$ mv.; a total voltage change of 65.0 mv. This is preceded by an unchanged auricular action potential. Following record *a* a ligature was tied tightly between the auricles and ventricle, resulting in ventricular stoppage. Record *b*, taken immediately after, shows only the small auricular component on the upper curve. In record *c* the quiescent ventricle was stimulated mechanically and its contraction was associated with the development of an injury activity potential with a total voltage change of 57.0 mv. An auricular action potential component is apparent on the ascending limb of the injury potential curve.

Figure 9 shows the results from a similar experiment but dealing with an auricular injury potential. A suction electrode was placed on the left auricle of a turtle's heart in situ and the circuit completed by a lead from the ventricle. Record *a* shows the normal action potentials of the auricle and ventricle (upper curve) before suction was applied. The lower tracing is a reference curve from

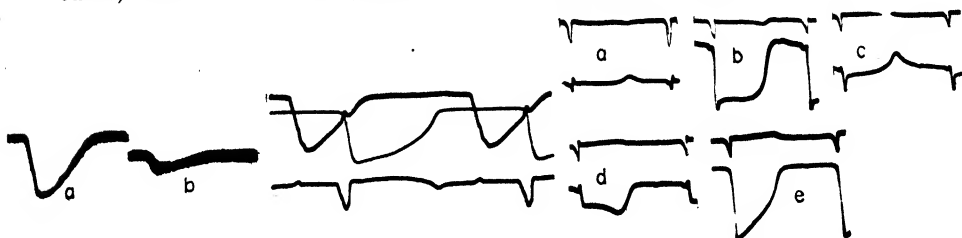


Fig. 11

Fig. 12

Fig. 13

Fig. 11. Injury potential from a sinus strip. For description see text.

Fig. 12. Simultaneous recording of injury potentials from an auricle and ventricle of a turtle heart. For description see text.

Fig. 13. Comparison of injury potentials from an injury due to a burn and an injury due to suction. For description see text.

the ventricular apex and pelvis. Record *b* shows the 4th and 5th cycles after a negative pressure of 200 mm./Hg was applied to the suction electrode. The auricular injury potential is completely formed in the fifth cycle and shows a resting potential of -17 mv. changing to a positive potential of $+18.0$ mv. during auricular activity. There is a large diphasic ventricular action potential component superposed. The injured region was now ligated off from the remainder of the auricle and following this record *c* was made. The injury potential of the auricle is reduced greatly, the small residue remaining probably arising from the site of the ligature. The ventricular action potential persists. In record *d*, the region of the right auricle containing the injury was stimulated mechanically. An injury potential with a total voltage change of 22.0 mv. results showing a superposed ventricular action potential.

Similar experiments have been carried out using automatically beating sino-auricular or sinus strips from turtle hearts. Figure 10 gives the results from an experiment of this type. A suction electrode was placed in contact with the auricular end of a sino-auricular strip, the circuit being completed by a wick

ment of a positive potential ending in a sharp peak; 3, decline in the positive potential from its maximum peak with a nearly constant potential until near the end of systole; 4, a rapid return to a state of negative potential, and 5, a slow decline of the negative potential until the next period of cardiac activity begins. Record *c* of figure 13 was made from the burned region 130 minutes after the injury was produced. The injury potential has markedly declined. A ventricular T wave has developed and the curve does not differ greatly from the normal action potential. Record *d* was made one minute after the same region was reburned. The curve obtained again resembles those frequently encountered in the development of or subsidence of a suction injury. Record *e* is a suction injury from a neighboring region of the same ventricle. The injury potential curve is monophasic and uncomplicated. It shows a resting injury potential of -14.0 mv. rapidly changing to $+20.0$ mv. with the onset of activity.

The variability and degree of departure of potential curves from burned areas from the uncomplicated monophasic type would appear to depend on the uniformity and extent of burn and whether or not the electrode made contact only with a uniformly injured area. It is more difficult still to obtain a crushed area in which the injury is uniform and in our experience the injury potential curves obtained from such areas are even more variable than those from burned areas.

DISCUSSION. The curve of potential change from an injured region during activity is basically monophasic and with smooth contour. It represents a condition during the resting condition of a negative potential with respect to uninjured resting muscle and rapidly develops a positive potential with respect to the same reference with the onset of activity. It declines smoothly and gradually from this maximum positive potential to return to the negative potential of rest. It thus consists fundamentally of only two parts, the rapid obliteration of the negative potential of rest and its replacement by a positive potential and a slower return to the resting state of negative potential. Modifications of the basic curve frequently encountered would appear to be due to two principal causes, incorporation within it of action potentials from regions of uninjured muscle and incomplete or non-uniform injury. The former is expressed by interruptions or added waves of potential change occurring before or during the rapid transition from the negative to positive potential state, while the latter affects the subsequent contour of the curve. The influence of action potentials from uninjured regions may be in large part avoided by the use of unipolar rather than bipolar leads and especially by the use of differential or coaxial leads as described. The deformations occurring later in the curve are absent in the uniform injuries produced by suction if the suction is sufficient. They are nearly always present in injuries produced by local burning or crushing. Rosenblueth, Daughaday and Bond (9) recognize seven separate excursions consisting of at least four components which they regard as integral parts of the injury potential resulting from crushing or burning. Their curves were recorded by bipolar leads, all show the inclusion of action potentials in their initial phases and later excursions which we have characteristically found in curves from regions exposed to insufficient suction or in which the injury potential is reversibly returning to a

normal action potential following the release of suction. These workers state that a "strictly monophasic" curve is seldom if ever obtained in injury potential curves and suggest that the response be called monotopic instead of monophasic.

In a series of papers Unghváry and Obál (12) have described injury potentials derived from the auricles and ventricles of the dog's heart and from the various chambers of the frog's heart. While using only a single channel recorder they were able to show that the injury potentials arise from the particular heart chamber in which the injury was present. In complete auricular-ventricular heart block with injuries on the auricle and ventricles monophasic injury potentials were recorded at the time of activity of the particular chamber. They also state that when two injuries are present on the same heart chamber and these two injured regions are led to the recorder, a normal action potential electrogram is obtained. From our work it is obvious that this would result only if the injury potentials from the two regions were identical in time of onset, voltage and contour, which in our experience is rarely if ever obtained (cf. records *a* and *d* of fig. 5 and fig. 6). In their last paper to which reference is made they employ a quadrant electroscope to record the potentials from injured regions and come to the final conclusion that the injury potential does nothing more than to produce a divergence of the zero line of the recorder and does not interfere with the recording of the normal action current. Their conclusions appear contradictory and contrary to the experimental results obtained.

The principal arguments that have been used by those investigators who contend that the potential change during activity existing between an injured and uninjured area arises at the uninjured region are based on two principal observations, the changes produced by changing the position of the electrode on uninjured tissues (the so-called different electrode) and the comparative effects of various influences applied to the injured and uninjured regions respectively. The experiments of Unghváry and Obál (12) in which the "different" electrode was placed on a cardiac chamber other than that containing the injury and the repetition and extension of these experiments reported in the present work show clearly that the position of the electrode on uninjured tissue does not affect the fundamental injury curve but produces changes resulting only from the inclusion of normal action potential from uninjured regions.

A number of groups of workers (1) (9) have reported that the injury potential is changed as regards onset and contour with different locations of the electrode on uninjured regions of the same heart chamber in bipolar leads, while changing the location of injury has little or no effect. We believe that these results are due to the inclusion of action potentials before or in the early part of the injury potential, which is commonly encountered in bipolar leads, particularly when the injury potential itself shows variations from the strictly monophasic type or is of relatively low potential. H. C. Wiggers (13) has shown in the dog's ventricle that when a monophasic injury potential is produced by suction, moving the electrode on the uninjured region to different points of the ventricles resulted in curves which were nearly simultaneous in onset, differing at most by 17 msec. The curves shown have sharp onsets and little or no evidence of inclusion of action

potential components. We have on a number of occasions repeated this experiment on both dog's and turtle's hearts and have found that with the exception of curves, the onset of which is distorted by action potential components, the onset is within the limitations of physiological variations in the same curve at different times and within the limits of error in measurement of the curves. On the other hand it is apparent that the onset of activity as indicated by recording local fractionate contraction or differential potential curves differs by as much as 0.12 second in the turtle ventricle (14) and by as much as 0.03 second in the dog's auricle (15) and ventricle (16). It has been further shown that the onset of the injury potential curve from different regions of injury bears a nearly constant relation to the onset of fractionate contraction and the other electrical curves (unipolar and differential) which signal this occurrence (17).

A large number of workers have attempted to determine the origin of the potential change in bipolar leads from an injured and an uninjured region of cardiac muscle by submitting the muscle under one or the other leads to the action of various chemical substances, changes in temperature or other influences. No attempt will be made at this time to review this work in detail. In most cases it has been found that the application of various chemicals, such as KCl, CaCl₂ and HCl, produces a greater alteration in the potential curve when applied to the uninjured region than when applied to the injured region and this finding has been used to support the view that the active potential change resides in the uninjured region. It is to be noted, however, that these substances in themselves are capable of producing injury. When applied to an injured region they would be expected to affect only the degree of injury while if applied to a previously uninjured region the bipolar lead becomes in effect the potential between two injured regions, which may profoundly modify the potential curve (cf. fig. 5, record d). It has been suggested that the release of potassium in muscle injury may play an important rôle in the production of the injury potential (18). The effect of local heating has been studied by several workers. Schutz and Lehne (10) regarded this as a crucial experiment in the interpretation of the origin of injury potentials. Ventricular strips were prepared from frogs' hearts mounted on cork and kept chilled by ice. One region was injured by crushing, electrodes connected to this and to an uninjured region and the strips were stimulated by shocks from an induction coil. Local warming, by focusing the beam from an arc light on the uninjured part of the strip, decreased the duration of the monophasic injury curve but was ineffective when applied to the injured region. We have repeated this experiment except the preparation used was a large intact turtle ventricle beating spontaneously *in situ*. The effects of local heating on the form and magnitude of the injury potential were greater on local heating of the initially uninjured area than on the injured region, but they occurred only if sufficient heat was applied to produce injury and result in the substitution of two injured areas from the previous state of one injured and one uninjured region. The decreased duration of the injury potentials reported by Schutz and Lehne may well have resulted from the fact that with the small heart preparation used the heating affected the strip as a whole resulting in decreased duration of the contractions.

SUMMARY AND CONCLUSIONS

By applying increasing increments of suction to a small region of heart muscle the development of the injury potentials of rest and activity has been observed. When completely developed the injury potential curve of activity is fundamentally monophasic in form and consists of only two parts: 1, a part in which the resting potential, negative with respect to uninjured resting muscle, is rapidly abolished and replaced by a potential positive with respect to the same reference, and 2, a slower and continued decline of the potential to the resting state. Modifications in this curve causing it to depart from this strictly monophasic type may result from two factors, inclusion of action potentials from uninjured regions of the muscle and from incomplete or non-uniform injury. The former occur most prominently in bipolar leads, are less often present in unipolar leads, and usually absent in coaxial leads. The latter effects are prominent during the development of suction injuries or during their subsidence and are always, in our experience, present in injury potential curves derived from burned or crushed regions.

It is shown by several experimental methods that the injury potential of activity is derived solely from the region of injury or the immediately contiguous tissue. The electrode on uninjured tissue contributes to the recorded curve only by the addition of action potential components arising from the region of the electrode or from normal heart tissue intervening between the two electrodes.

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PULSE REACTION TO PERFORMING STEP-UP EXERCISE ON BENCHES OF DIFFERENT HEIGHTS

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Stepping-up tests have been used for the clinical evaluation of physical fitness based upon cardiovascular response to a standard amount of exercise. Due mainly to simplicity in administration and the relative ease with which the workload can be controlled, tests of this type received a renewed emphasis during the war. This was particularly true in appraising the fitness of soldiers before return to duty following periods of hospitalization.

Of the several stool-stepping tests, there is little uniformity from test to test as to the height of step used. Consequently there is little known concerning the effect on the cardiovascular response of varying the height of the stool. In early experimentation with a test of this nature, Hambly (see Campbell (1)) used a block 13 inches in height. Later Campbell (1) and Tuttle (2) modified the Hambly test but retained the 13-inch stool. Schneider (3), for convenience, used an ordinary chair (18 inches in height) for performing the exercise. A test developed at the Harvard Fatigue Laboratory and reported by Johnson, Brouha, and Darling (4) has been used quite extensively in some military installations and requires the use of a bench 20 inches in height. Behnke, Welham, Pace, and White (5) reported upon work done at the Naval Medical Center, in which a test of cardiovascular response was developed calling for the use of an 18-inch "platform." In studies conducted upon patients convalescing from upper respiratory infections, Karpovich, Starr, and Weiss (6) have used the 12 and 20-inch benches. Master and Oppenheimer (7) used a two-step contrivance nine inches in height.

The present study was conducted to determine whether variation in the height of the bench (or stool) significantly affects the pulse reaction of normal subjects to the step-up exercise.

METHODS. Seventy-two healthy aviation students were tested in groups of three. Each subject was tested ten times, twice on each of five benches (12, 14, 16, 18, 20 inches in height). Two periods of exercises were used for each height of bench, one for 30 seconds and one for 60 seconds. In order to minimize the errors which might have been introduced by following a definite sequence of testing, each subject performed the ten exercises in a random order.

The step-up exercise was performed at the rate of 24 steps per minute (the cadence being set by a metronome) in the following manner: The subject stood before the bench and, at the starting signal, stepped upon it with his left foot, came to an upright position, and stepped down with his left foot. The complete cycle was considered a "step." Arms were held loosely at the sides throughout the exercise. Immediately after the conclusion of exercise, each sub-

ject sat down on the exercise bench and remained in this position until the pulse rate was taken.

Before each exercise, the subjects sat quietly for at least eight or nine minutes. The pulse rates were recorded prior to each period of exercise, immediately fol-

TABLE 1
Mean pulse rates for 60 second intervals at times indicated. n = 72

BENCH HEIGHT	DURATION OF EXERCISE	TIME OF PULSE RECORD			MEAN ALL TIMES
		Before test	Immediately after	One minute after	
<i>inches</i>	<i>seconds</i>				
12	30	75.0	90.0	70.0	78.4
	60	75.4	95.2	69.6	80.0
		—	—	—	—
	Mean	75.2	92.6	69.8	79.2
14	30	74.0	91.6	68.6	78.0
	60	74.4	100.8	71.2	82.2
		—	—	—	—
	Mean	74.2	96.2	69.9	80.1
16	30	74.4	96.4	69.4	80.4
	60	74.4	106.2	71.4	84.0
		—	—	—	—
	Mean	74.4	101.3	70.4	82.2
18	30	73.6	99.2	69.2	80.8
	60	73.0	110.4	72.4	85.2
		—	—	—	—
	Mean	73.3	104.8	70.8	83.0
20	30	74.0	103.6	70.2	82.6
	60	73.4	115.8	74.2	87.8
		—	—	—	—
	Mean	73.7	109.7	72.2	85.2
Mean	30	74.2	96.2	69.5	80.0
	60	74.0	105.6	71.8	83.8
		—	—	—	—
	Mean	74.1	100.9	70.6	81.9

Note: The standard error of any mean pulse rate (\bar{x}) which is based upon the 72 records from 72 subjects may be taken as $s_{\bar{x}} = \pm 0.48$. This is computed from $16.052/72$, where $s^2 = 16.052$ is found in the analysis of variance.

lowing exercise, and beginning one minute after exercise. While the subjects were in a sitting position, pulse records were taken by three experienced technicians for periods of 30 seconds and multiplied by two before recording. Throughout this study the "one minute after exercise" pulse rate refers to the pulse rate taken during a 30-second period beginning one minute after exercise. This procedure gave a total of 2160 pulse records.

A bench, 12 inches in height, eight feet in length, and ten inches wide, was used as the basic bench for this study. This bench was readily increased to the desired heights by placing the bench legs upon recessed blocks. The height of the bench was adjusted by an assistant during the time in which the resting pulse was being taken prior to each exercise period.

RESULTS. An examination of the mean pulse rates (table 1) shows that they were relatively uniform before exercise (average = 74.1). Immediately after exercise, pulse rates increased to 90 or more beats per minute. By one minute after exercise, pulse rates on the average returned to pre-exercise rates or lower (average = 70.6). The variability observed in the pulse rates is such that the pulse rate for any subject may be considered as typical for normal subjects unless the rate differs by 24 or more beats per minute from the averages given in table 1. The pulse rate of a single subject taken one minute after the step-up exercise should return to a level within 10 beats of his own pre-exercise level, where 10 beats is approximately 2.5 times the standard error of a single determination.

As shown in figure 1, the magnitude of the difference between the pre-exercise pulse rate and the pulse rate immediately after exercise is directly related to the height of the bench and the duration of exercise. For 30 seconds of exercise, the increment in pulse rate is 15.0 beats per minute on the 12-inch bench, and this increment grows rather steadily to 29.6 beats per minute on the 20-inch bench. These increments are plotted in figure 1A where it may be seen that in general the increment grows at the rate of 3.7 beats for each two-inch increase in the height of the bench. For 60 seconds of exercise, on the other hand, the increments in pulse rate vary linearly from 19.8 beats per minute on the 12-inch bench to 42.4 beats per minute on the 20-inch bench. This is an average rate of growth of the increment of 5.6 beats for each two-inch increase in the height of the bench (fig. 1B).

A comparison of the increments for the 30-second period of exercise with the increments for the 60-second period of exercise shows that the differences between the increments are not constant (fig. 1C). For the 12-inch bench, the difference is 4.8 beats per minute. The difference grows, somewhat irregularly, to 12.8 beats per minute for the 20-inch bench. The rate of growth on the average is 1.9 beats for each two-inch increase in the height of the bench.

The sequence of testing the subjects did not introduce a systematic bias. Before the first exercise, the average pulse rate was 72.4 per minute. The average fluctuated slightly and erratically to 73.4 per minute before the second test, and then to 75.4, 74.7, 75.1, 74.6, 74.0, 74.4, 74.4, and 73.2 on the succeeding tests. The interval of eight or nine minutes between tests is therefore regarded as adequate.

The study was designed so that the methods of the analysis of variance (8) were applicable in testing for the significance of the various factors involved in the experiment. The complete analysis is summarized in table 2. It may be seen that the analysis consists of identifying the effects of several factors, alone and in combination. Each assertion that has been made in the discussion of the results is supported by evidence in table 2. For example, the statement that

the increments in pulse rates are related to the height of the bench is based upon the fact that the interaction of "benches" with "times" is statistically significant. The $B \times T$ term in the analysis of variance has 8 degrees of freedom and a mean

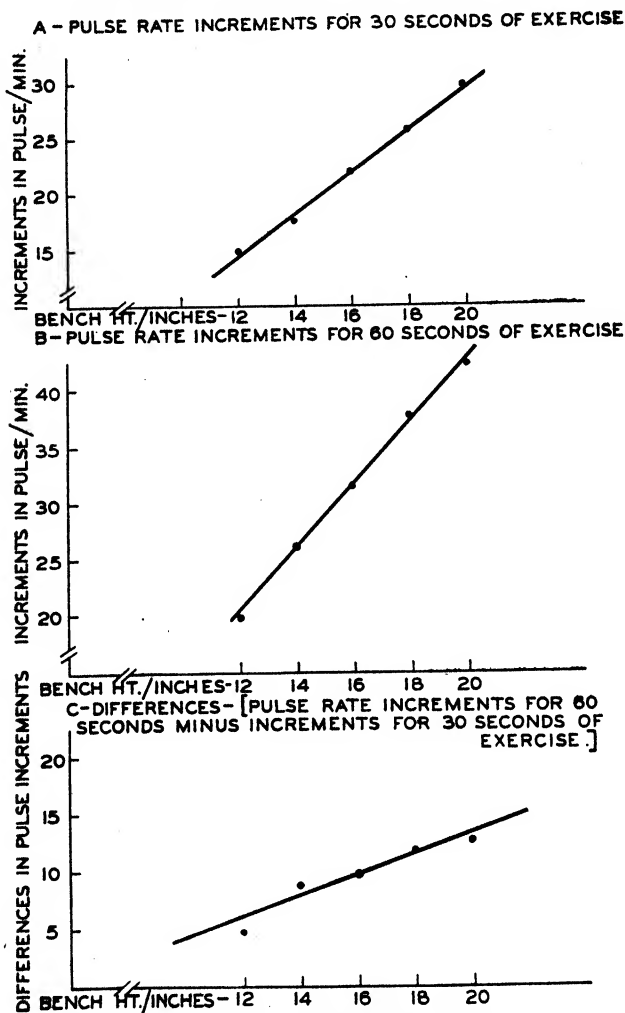


Fig. 1. Increments in pulse rates following step-up exercise

square of 2,187.32. This mean square is 136.26 times larger than the remainder mean square which is taken as the estimate of uncontrolled experimental variation. The ratio $F = 136.26$ greatly exceeds $F_{0.01} = 2.55$, the value which F is expected to exceed no more often than once in 100 times if there were no relationship between the height of the bench and the pulse change. The apparent

conclusion therefore is that the height of the bench does affect the change in pulse rate during exercise.

TABLE 2
Analysis of variance of pulse rates

SOURCE	DF.	MEAN SQUARES	F	F _{0.01}
Total.....	2,159			
Primary effects				
Individuals, I.....	71	1,749.24	108.97	1.47
Benches, B.....	4	2,441.64	152.11	3.36
Times, T.....	2	197,658.96	12,313.67	4.66
Durations, D.....	1	8,127.84	506.34	6.70
Sequences, S.....	9	391.16	24.37	2.46
First order interactions				
I × B.....	284	42.44	2.64	1.32
I × T.....	142	130.32	8.12	1.42
I × S.....	71	65.64	4.09	1.47
B × T.....	8	2,187.32	136.26	2.55
T × D.....	2	4,556.24	283.24	4.66
B × D.....	4	189.28	11.79	3.36
Second order interactions:				
I × B × T.....	568	17.84	1.11	1.24
I × B × D - S.....	275	29.52	1.84	1.32
I × T × D.....	142	29.56	1.84	1.42
B × T × D.....	8	90.12	5.61	2.55
Remainder.....	568	s _e ² = 16.052		

TABLE 3

Minimal difference that must exist between comparable means for the difference to be significant with $P < 0.01$

MEANS TO BE COMPARED	NUMBER OF RECORDS ON WHICH EACH MEAN IS BASED, k	STANDARD ERROR OF DIFFERENCE, s_{1-2}	LEAST SIGNIFICANT DIFFERENCE
Duration means.....	1,080	0.172	0.44
Bench means.....	432	0.273	0.70
Time means.....	720	0.211	0.54
D × B means.....	216	0.386	1.00
D × T means.....	360	0.299	0.77
B × T means.....	144	0.472	1.24
B × D × T means.....	72	0.668	1.78

The variances in the subclasses of the data were found to be homogeneous upon testing by Bartlett's (9) method.

The standard error of a single determination was found to be $s_e = 4.01$, where

$s_e^2 = 16.052$ is the error or remainder mean square in table 2. If it is desired to estimate the statistical significance of the difference between any two comparable means, the appropriate standard error for the difference may be obtained from the same error mean square. The square root of twice this error mean square divided by the number of records on which each mean is based gives the standard error of the difference, s_{1-2} , between any two comparable means. That is, $s_{1-2} = \sqrt{2s_e^2/k}$ where k is the number of pulse determinations on which each mean is based. The summary in table 3 shows the minimal differences that must exist for the difference to have a probability of less than one in a hundred of arising by chance alone. For example, the average pulse rate of 75.2 before exercise on the 12-inch bench may be compared with the average of 74.2 before exercise on the 14-inch bench. These means are each based on 144 records. Their difference is 1.0, which being less than 1.24 is regarded as non-significant.

This study indicates that, on the average, the pulse rate of a normal male subject, after participating in 30 or 60 seconds of the step-up exercise on benches of 12 to 20 inches in height, when taken for 30 seconds starting one minute after exercise, should be no more than 10 beats greater than his pre-exercise rate. The pulse rate taken immediately after exercise is definitely affected by the height of the bench and the duration of exercise.

Not all subjects responded to the variations in height of the bench and duration of exercise in precisely the same fashion. Upon repeating this sequence of tests with a single subject, it can not be anticipated that his response will be exactly like the averages shown in table 1.

SUMMARY

In normal subjects, following the step-up exercise, for periods of 30 or 60 seconds, on benches of 12 to 20 inches in height, the pulse rates taken for 30 seconds beginning one minute after exercise are practically the same for the various heights of the benches and the durations of exercise.

The pulse rate for the 30 seconds immediately following the stepping-up exercise depends upon the height of the bench and the duration of the exercise. After 30 seconds of exercise, the average increment is 3.7 beats per minute greater for each additional two-inch increase in the height of the bench. After 60 seconds of exercise, the increment becomes 5.6 beats per minute greater for each additional two-inch increase.

The height of the bench and the duration of the step-up exercise as used in this study do not significantly alter the pulse rate if the pulse record is taken one minute after exercise.

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THE ACTION OF CARDIAC EJECTION ON VENOUS RETURN

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The functioning of many of the organs of the body probably depends very little on their situation and environment but this is not true of the heart. Surrounded by sacks of air in a chamber with flexible walls, its action as a pump differs considerably from that which would attend residing like the brain within a rigid shell or like the biceps under a loose covering of skin. The present purpose is to discuss some of the problems arising from the placing of the heart in relation to its size, the lung size and the ambient pressure as they affect the pumping of blood from and to the chest.

It was pointed out by Donders in 1853 that venous return to the chest is aided by the sub-atmospheric pressure existing between the pleural surfaces of the lungs and thorax. This pressure deficit is maintained by the metabolic processes in the surrounding tissues and made manifest by the elastic recoil of the lungs which, since they are normally stretched, pull on all the surfaces they touch. The pressure difference between the extra- and the intrathoracic veins, amounting ordinarily to about 5 mm. of Hg, assists in refilling the large veins in the chest and the right auricle and ventricle each time they are partially cleared by the heart's action. This mechanism can be considered a venous pump but the left ventricle provides the energy since it must keep ejecting arterial blood from the chest against the same pressure difference or the venous pump will stop. This is one way in which the left ventricle in effect pumps blood into the chest as well as out but there is another of some importance which seems to be much less well understood, although its existence was recognized by Buisson in 1861 and Voit in 1865.

The chest is a closed chamber except for the trachea, the aorta, the large veins and other smaller vessels. In man at rest with each heart beat about 60 cc. are ejected from the left ventricle and subsequently, in consequence, from the chest by way of the aorta and other arteries. Since the lungs surround the heart and contain about 3000 cc. of air, the ejection of each stroke volume of the ventricle tends to increase the lung volume to 3060 cc. and to reduce the intrapulmonary pressure by 60/3060 atmospheres or about 15 mm. of Hg. This theoretical lowering of pressure is not actually realized, however, because some air enters the trachea if the glottis is open, venous blood returns to the chest, the chest wall collapses inward, and the diaphragm probably rises. Reviews of the early observations of these movements are given by Luciani (1911) and Klewitz (1918).

Only about 1 cc. of air enters the trachea with the heart beat when the breath is held in man (Hamilton, 1930) and less than $\frac{1}{2}$ cc. in the dog (Klewitz and

Baumm, 1921). This is less than might be expected from the low resistance of the trachea and bronchi to respiration, but it must be remembered that the area of the chest wall and diaphragm is very large relative to the cross section of the trachea. In consequence the total force exerted on these surfaces by any change of pressure in the lungs is correspondingly large and only a very small movement is required to bring about an appreciable change of volume. Failure to realize that the chest wall moves relatively easily has led Hamilton (*loc. cit.*) and others

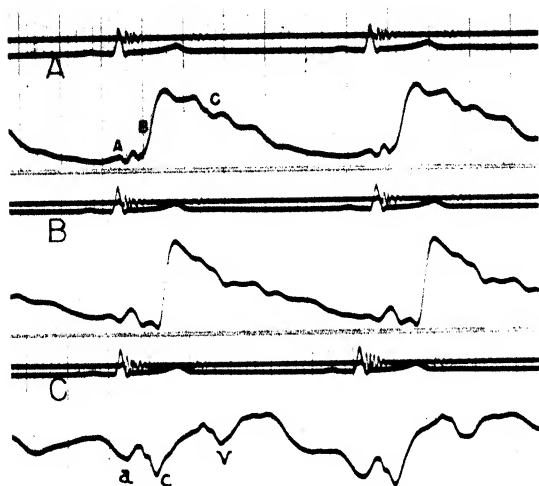


Fig. 1

Fig. 1. A, B and C respectively, are records of the changes in circumference of the chest, abdomen and neck in response to the heart beat. Recording was done with the Sanborn Tri-beam Stethocardiette using a pneumograph with the pulse recorder. Respiration was suspended. Heart sounds and lead II of the electrocardiogram were recorded simultaneously. In all cases upward movement denotes decrease of volume. The neck record resembles that of a venous pulse inverted. The *a*, *c*, and *v* waves are indicated.

Fig. 2. Changes in pressure in a closed chamber of 1200 liters capacity due to the heart beat of a subject seated therein with respiration suspended. In the upper tracing the breath was held on normal expiration while in the lower it was held on maximal inspiration. Upward movement denotes increase of volume of the subject with the heart beat. It amounts to about 2 cc. in the upper tracing. There is a more complicated cycle of decreased, increased then decreased volume in the lower tracing.

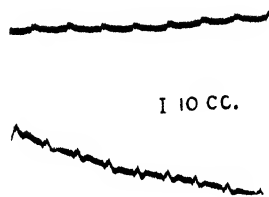


Fig. 2

to the erroneous conclusion that the venous return differs from the arterial outflow from the chest by an amount equal to only that small volume of air which may be moved through the trachea by the heart beat. Direct calibration of the change in chest volume with the heart beat (Blair and Wedd, 1939) leads to a very different conclusion and makes much clearer the effect of ejection on the surrounding tissues.

The chest wall collapses sufficiently to reduce the lung volume by about one-half the ventricular ejection or 30 cc. The collapse is easily recorded by a suit-

able device to give a curve like A in figure 1. This curve is a measure of the change in circumference of the chest at a given level. It is calibrated in terms of volume by introducing or removing air from the lungs by way of the mouth in sufficient amount to give a similar excursion (Blair and Wedd, *loc. cit.*). This procedure is considered completely valid because the lungs are elastic and fill the space in the chest not occupied by other organs and vessels. In consequence the removal of blood from a contiguous vessel may be expected to affect the pressure in the lungs in just the same way as the removal of an equal volume of air from within. In turn the pressure change whether produced by blood leaving the chest or by the removal of an equal volume of air may be expected to move the diaphragm, venous blood and the chest wall in the same way. In accord with this hypothesis the chest wall movement as calibrated is an index of the change in air capacity of the chest whatever its cause.

Curve B in figure 1 records the collapse of the abdomen at the level of the umbilicus following ventricular ejection. This record indicates that during systole the abdomen loses more blood to the chest as a result of the heart beat than it gains in both arterial blood from the chest and venous blood from the periphery. There is a complicating factor, however, in that part of the collapse of the abdomen is most probably due to lifting of the diaphragm. The contribution of the diaphragm is difficult to estimate but it is plain since both the chest and abdomen collapse that the trunk as a whole loses volume following ventricular ejection. Consequently the circulation has a tidal quality in that blood flows to the periphery following systole and ebbs during diastole. This is obvious also, of course, from plethysmographic records of the limbs which show increase of volume with the pulse.

Since the chest decreases in air capacity by only 30 cc. when 60 cc. of arterial blood are ejected, the other 30 cc. must be made up in some other way. Some of this, but probably not a large fraction, can be ascribed to ejected arterial blood still distending the thoracic arteries; the remainder must be ascribed to inflow of venous blood. It appears therefore that venous inflow is more rapid during ejection than in the rest of the cycle. For example, if the heart cycle is taken to be 0.8 sec., rapid ejection, as indicated by rapid collapse of the chest wall, lasts about 0.16 sec., or $\frac{1}{5}$ of this time. Consequently if the venous return were constant throughout the cycle about $\frac{1}{5}$ of 60 cc., or 12 cc., would return during rapid ejection. The difference between this quantity and the 30 cc. actually returned is attributable in part to aspiration as will be shown later.

It will be seen from these considerations that the situation of the heart has an important influence on its action. If it were placed just under the skin, the activity of the ventricle would be devoted to the pumping of arterial blood only, because the skin would not support a sub-atmospheric pressure analogous to the intra-pleural pressure. Also it would collapse too readily to support a lowered pressure due to cardiac ejection with consequent aspiration of venous blood. On the other hand, if the heart were situated within a rigid case like the cranium, the ventricle would be forced to pump in venous blood at the same rate as it ejected the arterial blood or else it would tend to create a vacuum and have to

work against atmospheric pressure. With the heart in the chest, the air in the lungs sets an upper limit of a small fraction of an atmosphere on the partial vacuum which the left ventricle can produce by ejecting and even this limit is probably never very closely approached because venous blood moves readily and the chest wall collapses in approximately equal measure. Thus its situation in the thorax enables the heart to act as a venous pump to a limited extent while at the same time retaining a wide margin of safety for its more onerous duty as an arterial pump.

An interesting consequence of the aspirating action of cardiac ejection is that the volume of the body increases temporarily with each heart beat. This is due to the partial vacuum created in the chest by the failure of venous inflow and collapse of the chest wall to keep pace with arterial ejection. Figure 2 shows the variation of body volume with the heart beat obtained by recording the pressure changes in a closed chamber of about 1200 litres capacity in which a subject is seated and is holding his breath. Both records are on the same subject but in the upper the breath was held after normal inspiration while in the lower the chest was highly inflated. The latter is more complex. In these cases the subject's increase in volume with each systole is 2 cc. corresponding to a partial vacuum of about $2/3000$ atmospheres if a 3000 cc. chest volume is assumed. Since the aspirating action must be mediated through the air of the lungs the above pressure change, less than 1 mm. Hg, is all that can be devoted to promoting venous return. Consequently under these conditions the negative intra-thoracic pressure which is more than 5 times greater than the aspirating pressure is much the more important. As will be shown later however this is probably not always true. Meanwhile the work which is done and could be done by the left ventricle to promote venous return will be calculated.

That part of the work of the left ventricle available for direct promotion of venous return may be compared with the part devoted to raising arterial pressure. Taking the average arterial pressure as 100 mm. Hg, the approximate work of the ventricle in raising a stroke volume of 60 cc. from zero to this pressure is $60 \times 980 \times 135$ or about 80×10^6 ergs. Since however, the intra-thoracic pressure is -5 mm. Hg, further work is required to expel the stroke volume against it. This amounts to $60 \times 980 \times 6.5$ or about 3.8×10^6 ergs. As was mentioned above this is the price paid by the left ventricle for the promotion of venous return by the subatmospheric intra-thoracic pressure. It is about 5 per cent of the energy imparted to arterial blood at ordinary blood pressures.

The greatest work that could possibly be done by the left ventricle in aspiration is that which would be done in expelling the stroke volume against the partial vacuum created in a rigid chest with venous return arrested. Assuming a 3000 cc. lung volume and 60 cc. stroke volume this work would amount to $760 \left(3060 - 3000 \left(1 + \log \frac{3060}{3000} \right) \right) \times 13.5 \times 980$ or 6.03×10^6 ergs. Under the conditions postulated this work would be available in the form of a partial vacuum to act on the venous blood.

The work actually done is difficult to estimate since it involves components

which move the chest wall and diaphragm as well as the blood. Considering the venous blood alone about 30 cc. returns during $\frac{1}{2}$ sec. of arterial ejection. If the cross section of the vein is 2.5 cm^2 , the column entering is 12 cm. long with velocity of 72 cm. per sec. The kinetic energy is therefore 15×72^2 or about 8×10^4 ergs. This estimate is too small because more peripherally situated blood will also be accelerated but even if it is doubled, the $\frac{1}{2}$ part of it (3×10^4 ergs) is all that is attributable to aspiration as compared with the part attributable to the negative intra-thoracic pressure. This is only 5 per cent of 6×10^5 ergs, the total potential aspirating work.

The lower limit of the work done by aspiration in moving the chest wall can be estimated from the pressures (relaxation pressures) required to hold the chest statically at various inflations. According to these data a change in chest volume of 30 cc. requires about 1×10^4 ergs of work.¹ If this is doubled to allow for kinetic factors and added to the work expended on venous return as estimated above, it appears that about 5×10^4 ergs or $\frac{1}{2}$ only of the potential aspirating work of the left ventricle is actually used. Consequently it may be concluded that under normal conditions the properties of the heart-chest system are such as to keep the effective aspirating forces low. Aspiration requires much less work of the ventricle than does the negative intra-pleural pressure and is much less important in promoting venous return. The achievement of this result depends on various physical properties of the system which may be discussed.

It is easily seen that the relative sizes of the heart and chest are very important in determining the factors under consideration. For example in man, if the lung volume were reduced to one-half, i.e., to 1500 cc., and the stroke volume of the ventricle were doubled to 120 cc., the potential lowering of intra-thoracic pressure with the heart beat would be $\frac{1}{2}$ atmospheres or about 60 mm. of Hg.

If under these conditions the intra-pleural pressure remained the same with equal effect in promoting venous return there would be about 90 cc. deficit of blood in the chest following each ejection to be compensated for by collapse of the chest wall. This volume which is a substantial fraction of the tidal respiratory volume would be reduced of course, in such measure as extra venous return was promoted by aspiration. Failure of the chest wall to collapse easily would throw a larger burden on the ventricle. It is significant in this regard that the weights of both the heart and lungs vary roughly with the weight of the animal although in the larger animals these organs are relatively somewhat smaller. Brody and Kibler (1941), for example, have calculated from empirical equations fitted to data from many species of adult animals, a table relating the weights of various organs in percent of the weight of the animal. It will be seen from the following examples, including the extremes, that the large animals have relatively somewhat smaller hearts and lungs but the ratio of heart weight to lung weight is nearly one-half and quite constant.

¹ Dr. Hermann Rahn—personal communication.

WEIGHT OF ANIMAL	HEART WEIGHT	LUNG WEIGHT	RATIO
	<i>per cent</i>	<i>per cent</i>	
10 grams	0.634	1.200	0.53
1 kgm.	0.588	1.131	0.52
100,000 kgm.	0.487	0.965	0.50

Clark (1927) points out divergences from the rule in young animals and certain species but they are not large.

The stroke volume of the heart is probably about proportional to the weight as may be deduced by the following reasoning. If the heart is assumed to be a spherical shell the muscle tension T in the wall will be related to the pressure developed P and the internal radius r by the relation $Pr = 2T$. Since the blood pressure does not change much with the size of the animal it may be considered constant. Therefore, the required muscle tensions in hearts of various sizes may be assumed to be given by $T = \text{constant} \times r$. The tension developed by a muscle, however, varies as its thickness; consequently the thickness of the heart wall will also vary as the radius and may be called kr . Thus the volume of the whole organ is $4/3\pi (r + kr)^3$, the internal capacity is $4/3\pi r^3$ and the weight is determined by the difference $4/3\pi r^3 [(1 + k)^3 - 1]$ which, it will be seen, is in proportion to the capacity and consequently to the stroke volume. This conclusion agrees with the observations of Clark (l.c.), table 25, that in a group of mammals ranging in size from the mouse to the horse the weight of the heart varies as the length cubed of the left ventricle.

The lungs on the other hand can probably be considered to be increased in size by the addition of cells of approximately equal volumes and wall thickness. Therefore in close approximation the volume will again be in proportion to the weight. Thus it may be concluded finally that the stroke volume of the heart remains approximately proportional to the lung volume, and that in consequence the potential lowering of pressure in the lungs due to ventricular ejection and its effect on venous return and the chest wall is independent of body size. This avoids certain difficulties but raises others. Because the blood flow of small animals is relatively high their hearts must pump much faster to provide adequate circulation. For this reason heart rates as high as 1000 per minute are found in small birds compared to 70 in man and 40 in the horse (Clark, l.c.). The very fast heart rates are probably relatively inefficient because muscle cannot exert high tension when it is contracting rapidly. Nature, however, appears to favor this solution of the problem rather than that of increasing the size of the heart relative to that of the lungs.

Another factor in addition to the relative sizes of hearts and lungs which determines the aspirating effect of arterial ejection is the pressure of air within the lungs. Assuming again a 3000 cc. lung capacity and a 60 cc. stroke volume it will be seen that at a pressure of $\frac{1}{2}$ atmosphere the potential lowering of pressure within the chest by the heart beat will be only $\frac{1}{2} \times 380$ mm. or about 7.5 mm. of Hg instead of 15 mm. In general the potential aspirating force will be in

proportion to the pressure of the air in the lungs providing the stroke volume is constant. In flying at high altitudes heights of 30,000 ft. are commonly attained where the pressure is about $\frac{1}{4}$ atmosphere and in consequence the aspirating force acting upon venous blood is $\frac{1}{4}$ of its normal value. The promotion of venous return by aspiration will be reduced correspondingly but probably this will be of no great importance.

It will be seen in connection with ambient pressure problems that laboratory investigations to determine the effects of high altitude by lowering only the proportion of oxygen in the air are perhaps not quite equivalent to lowering the total pressure as far as the circulation is concerned. It will be evident also that opening the chest widely as an experimental or surgical procedure eliminates all assistance to venous return by aspiration as well as by the negative thoracic pressure. It eliminates also, however, the expenditure of energy by the outgoing arterial blood in collapsing the chest wall.

Increased ambient pressure as encountered in diving and caisson work will have an effect opposite to that of high altitude. At pressure corresponding to a depth of 175 ft. of water the potential pressure lowering in the chest with cardiac ejection will approximate 100 mm. Hg, the average blood pressure of young subjects. If this partial vacuum were realized, the load on the left ventricle would be quite high. Since, however, the forces on the chest wall and the other tissues giving compensatory movements are increased correspondingly there is no reason to suppose that the relative movement of venous blood and other tissues will differ greatly from that at sea level when the body is surrounded by air. When the body is surrounded by water, however, the situation may be different because then any portion of the body wall can move only if it moves the contiguous water along with it. This will have the effect of making the body wall approach a rigid system as regards its response to quick changes of volume. Consequently the outgoing arterial blood will tend to create a greater vacuum and venous aspiration will be increased. It seems probable that under these conditions compensation will be provided to a considerable extent by the movement of air through the trachea if this is possible. The tidal quality of the circulation will probably be reduced also since it will be easier for the arterial and venous blood to exchange peripherally at equal rates.

Under this pressure, particularly when the breath is held, considerably more work may be done by the ventricle in its aspiratory action. It does not appear likely that this will ever become large enough to be a serious burden because even at this pressure only about 40×10^6 ergs of work would be necessary to remove 60 cc. of blood from a rigid chest of 3000 cc. capacity. This amount is still less than one-half the normal stroke work of the ventricle.

Those changes occurring in cardiac failure may be of considerably more importance than any ordinary variations of ambient pressure of lung volume or ventricular stroke volume. With accumulation of blood in the pulmonary circulation not only is the capacity of the lungs reduced but the resistance to change of volume is increased by the increased weight of the lungs and their reduced elasticity. On account of the reduced capacity the potential aspirating force

will be increased and on account of the resistance change compensatory movement of the chest wall will be more difficult. Consequently promotion of venous return may be expected to be considerably increased.

In mitral stenosis even in the absence of failure the venous return to the chest frequently appears to exceed arterial outflow during systole. This is indicated by the finding that in these cases the chest may expand during systole and collapse during diastole. Examples are given in figure 3 A and B in which the upper tracings are taken from the chest and the lower from the neck. Upward and downward excursions denote, respectively, collapse and expansion in all cases. It will be seen in A that the neck expands at about 0.8 sec. after the beginning of the Q.R.S. interval reaching a maximum at 0.14 to 0.16 sec. This excursion is initiated by the arterial pulse. Referring only to the first of each pair of coupled beats it will be seen that the chest begins to expand at about 0.15 sec. coincidentally with the beginning of collapse of the neck. It appears reasonable therefore to conclude that the chest is gaining blood rapidly at this time from the periphery. This inflow cannot be due to heightened venous pressure alone or it

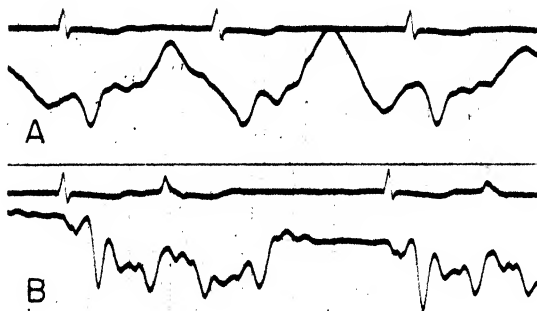


Fig. 3. A and B show respectively the volume changes of the neck and chest in a case with a clinical diagnosis of mitral stenosis. The chest volume increases with the heart beat rather than decreases as in the normal subject of figure 1. Recording was done with the Sanborn Tri-beam Stethocardiette. Upward movement is decreased volume in all cases.

would have taken place during the preceding diastole. It could possibly be due to the filling of a part of the venous system which had been pressed upon by the enlarged heart during diastole and released during systole. If such were the case, however, it would be expected that expansion would be prominent during isometric contraction whereas the first large movement is near the phase of maximum ejection. Consequently it seems most probable that this excursion is due to venous blood accelerated by aspiration. On the other hand there would seem to be no way in which any but very temporary net expansion of the chest could occur with aspiration alone. In cases such as those of figure 3 it is most probable that both aspiration and increased capacity are factors in the systolic expansion.

Figure 4 gives a somewhat different case in which the chest expands with iso-

metric contraction as a consequence presumably, of the apex thrust of the heart. During ejection, inflow and outflow are in approximate balance. The neck in this case expands in early and collapses in mid systole. No reason can be given for the expansion except that it is due possibly to tri-cuspid insufficiency.

In both figures 3 and 4 the chest collapses just beyond the T wave at the time of ventricular relaxation. Evidently venous blood is being lost from the chest at this stage. In A there is no important oscillation but in B the chest volume attains a minimum value from which it expands again rapidly. In A it appears that the heart must fill before or at the time of the loss of venous blood. In B it appears more likely that venous blood is lost from the chest before the heart is able to fill and it returns again as filling takes place. This inability to fill rapidly may be a consequence of tri-cuspid pathology.

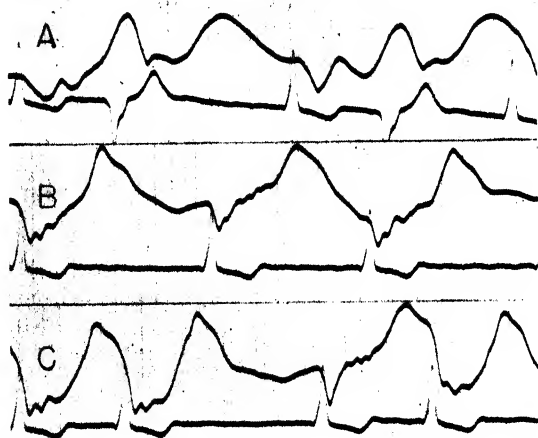


Fig. 4. A, B and C respectively, are records of volume changes of the neck, chest and chest with shorter diastolic intervals, in a case diagnosed as mitral stenosis. Recording was done with the Sanborn Tri-beam Stethocardiette. Upward movement indicates decrease of volume. C shows how chest expansion during systole may be exaggerated by a sort of resonance when the heart rate is appropriate.

Figure 4 C which is another chest tracing from the same patient as A and B shows that when the heart rate is in proper relation to the period of oscillation of venous blood the expansion of the chest during systole becomes relatively very large.

These examples suffice to show that aspiration may have very striking effects on the dynamics of venous blood flow in cases in which there are consequences of valvular damage. The importance of these changes in relation to their possible contribution to cardiac failure remains to be determined. It is evident, however, that the dynamics of the circulation in the chest are sufficiently different from normal to make their recording of diagnostic value.

SUMMARY

The mechanical relations between cardiac ejection, lung air pressure, chest wall movements and venous return are discussed. The excess of arterial outflow over venous inflow to the chest during systole tends to create a partial vacuum of about 15 mm. Hg in the lungs. Collapse of the chest wall, however, permits only a small fraction of this to be realized, about 0.5 mm. Hg according to derivations from measurements of transient increase of body volume with each heart beat. This partial vacuum will assist venous return but it is not nearly as important as the negative intra pleural pressure in performing this function. The relative sizes of cardiac stroke volume and lung volume determine the partial vacuum which tends to be created in the lungs by the heart beat and therefore the potential aspirating forces on venous blood and chest wall. The ratio of cardiac stroke volume to lung volume is approximately constant in warm blooded animals. Consequently, the relative mechanical effects of ejection are similar in animals of all sizes. Decrease of lung volume caused by accumulation of blood in the chest and the accompanying changes of elasticity may be expected to increase the aspirating action of arterial ejection. That this occurs is not proven directly, but it is shown that the normal mechanical relations may be greatly altered in valvular disease of the heart. In advanced mitral stenosis, for example, the chest may expand rather than contract during systole.

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FURTHER OBSERVATIONS CONCERNING EFFECT OF ADRENALECTOMY ON ALLOXAN DIABETES: PAIRED FEEDING EXPERIMENTS¹

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It has been shown that adrenalectomy ameliorates diabetes, causing a marked reduction or complete disappearance of symptoms in alloxan-treated rats (1). In 1936 Long and Lukens (2) found that cats which were depancreatized survived an average of four days. When depancreatized cats were adrenalectomized and given small amounts of adrenal cortical extract, however, they lived for 18 days. These writers explained the effect of adrenalectomy upon pancreatic diabetes as due to diminution of the production of glucose and acetone bodies rather than to resumption of normal carbohydrate metabolism. Later work by Long, Katzin and Fry (3) showed that the glycosuria produced in rats by pancreatectomy was stopped or greatly reduced by removal of the adrenals. Although data are given for food intake in certain of their experiments, these cannot be regarded as having followed the recognized procedures for paired feeding (4).

Since adrenalectomized rats show a characteristic loss of appetite and reduced food intake (5), the present study was made to determine whether the modification of diabetes in alloxan-treated rats, which follows adrenalectomy, is due to the absence of the adrenals, *per se*, or whether the lowered food intake was partially responsible. Alloxan-treated (125 mgm./kgm. body weight) male rats of the Long-Evans strain, which had been diabetic for 5 to 6 weeks were used in this paired feeding experiment. The diet fed consisted of:

Casein.....	350 grams
Crisco.....	100 grams
Wesson oil.....	100 grams
Sucrose.....	600 grams
Corn starch.....	600 grams
Yeast (Pabst).....	200 grams
Salt mixture (6).....	50 grams
Choline.....	400 mgm.
Haliver oil.....	4 cc.

Ten pairs of rats were selected from the group of diabetic animals, the pairing being based on similar blood sugar levels and comparable weight of the animals. Bilateral adrenalectomy was performed on one rat of each pair two days before the paired feeding was begun. Periadrenal fat was removed at the time of operation in an attempt to reduce accessory adrenal cortical tissue. An initial

¹This investigation has been made with the assistance of a grant from the Committee of Therapeutic Research, Council on Pharmacy and Chemistry, American Medical Association.

dose of $\frac{1}{4}$ cc. adrenal cortical hormone (A. C. H.)² was administered subcutaneously to these animals immediately after adrenalectomy. The amount of food eaten by the adrenalectomized animal in one 24 hour period determined the amount that was given to the control mate in the following 24 hours. Food allotments for the control rats were divided into halves and fed at 12 hour intervals. In this way, the control animals were always one day behind the adrenalectomized rats, and tests run for comparison of the two groups were made a day later for the controls than for the adrenalectomized rats. None of the rats showed acetonuria during the experiments.

As the study progressed, some of the adrenalectomized rats ate so little food that it was necessary to administer small doses of A. C. H. in order to keep them alive. Whenever an adrenalectomized animal failed to eat at least 4 grams of

TABLE 1

Average values for 7 pairs of rats showing the effects of adrenalectomy on alloxan-diabetes. Paired feeding experiments

CONTROL DIABETIC RATS						ADRENALECTOMIZED DIABETIC RATS					
Date	Body weight	Food 24 hr.	Blood sugar 24 hr.	Urine 24 hr.	Urine sugar 24 hr.	Date	Body weight	Food 24 hr.	Blood sugar 24 hr.	Urine 24 hr.	Urine sugar 24 hr.
	grams	grams	mgm. %	cc.	grams		grams	grams	mgm. %	cc.	grams
7-30	313	24.0	447	139	13.28	8-1	303	24.0	476	146	12.72
8-2						8-2	Bilateral adrenalectomy				
8-7	310	12.0	194	59	4.37	8-6	287	12.0	349	61	4.03
8-11	306	13.5	302	61	4.20	8-10	295	13.5	418	53	2.38
8-15	303	12.0	216	57	3.43	8-14	295	12.0	290	58	2.68
8-18*	300	0.0	136	24	0.25	8-18*	293	0.0	84	12	0.08
Liver glycogen at end of fast 875.6 mgm. %						Liver glycogen at end of fast 131.8 mgm. %					

* Fasted 24 hours.

the diet in a 12 hour period either $\frac{1}{8}$ or $\frac{1}{4}$ cc. of A. C. H. was given, depending on the severity of the anorexia. Even with this procedure one animal died four days after operation, another 14 days afterward, and a third died 15 days following operation. The seven adrenalectomized rats remaining at the end of the experiment had received an average total of $1\frac{1}{2}$ cc. of A. C. H. during the 16 day period, in addition to the initial dose of $\frac{1}{4}$ cc.

Daily determinations were made of changes in body weight, volume of urine, qualitative urine sugar, acetone bodies and food intake. Every fourth day quantitative blood sugar and urine sugar determinations were made by the Shaffer-Hartman-Somogyi micro and macro methods, respectively. At the end of 13 days of paired feeding, the surviving animals were fasted for 24 hours and then sacrificed in order to determine their liver glycogen. Approximately $1\frac{1}{2}$ grams of tissue were removed from both the right and left lobes of the liver for analysis by the method of Good, Kramer and Somogyi.

²Supplied through the kindness of Dr. F. G. Cartland of the Upjohn Co.

Data covering this experiment on both adrenalectomized and control alloxan-treated rats are presented in table 1. It will be noted that results obtained with the control animals closely paralleled those from the adrenalectomized series. In both groups there was a loss of body weight consistent with the reduced food intake, a steady decrease in the volume of urine, reduction of the quantity of sugar excreted in 24 hours, and a corresponding reduction in blood sugar readings. The blood sugar averages for the control animals following limitation of their food intake on August 7th were considerably lower than those of their adrenalectomized mates and remained so through August 15th. This is probably because some of the control animals consumed their food before the end of the 12 hour feeding period and consequently were fasting when the blood samples were taken.

After fasting for 24 hours the carbohydrate stores of the adrenalectomized animals were largely depleted. This is in agreement with the observations of Widström (7) and Lackey et al. (8). The blood sugar values were lower for the adrenalectomized animals and their average liver glycogen was only 15 per cent of that found in the controls (see table 1).

We have noted that our rats of the Long-Evans strain generally possess some accessory adrenal cortical tissue, which hypertrophies following bilateral adrenalectomy. In the present experiment five out of the seven adrenalectomized rats had small amounts of accessory adrenal cortical tissue when they were examined 16 days after the operation. Because this strain of rats has accessory cortical tissue, the animals in this experiment did not receive supplementary salt or desoxycorticosterone acetate and only very small doses of A. C. H., compared to the amount which is required for maintenance of life in certain other strains of rats.

The present study differs from the earlier investigation (1) in that the animals were diabetic for a longer period of time before adrenalectomy, and they were fed on a synthetic diet and studied under paired feeding conditions. The data for the diabetic adrenalectomized animals are very similar in each of the two experiments. Both show a marked reduction in blood sugar and glycosuria. However, by paired feeding, it has been possible to show that amelioration of the diabetes which was seen in earlier experiments following adrenalectomy (1) was almost certainly associated with a lowered food intake.

SUMMARY

Rats of the Long-Evans strain made diabetic with alloxan were adrenalectomized and studied under paired feeding conditions. In the present experiment, the amelioration of diabetes which occurs following adrenalectomy was found to be associated with a lowered food intake, with resulting decreases in urine and blood sugars, rather than to adrenalectomy *per se*. Following a 24 hour fast, however, the carbohydrate stores of the adrenalectomized diabetic animals were largely depleted; in fact, the liver glycogen levels of these animals averaged only 15 per cent of that found in the control diabetics.

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RELEASE OF PHOSPHATE BY THE BRAIN UPON STIMULATION

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The contraction of striated muscle in the frog produced by direct or indirect stimulation causes a loss of phosphates according to the experiments of Embden and Adler (1922), Behrendt (1922), Weiss (1922), Embden and Lange (1923) Plant and Cardoso (1923) and others. According to Eggleton (1929) this liberation of phosphate is caused by the hydrolysis of phosphocreatin to yield free phosphoric acid, to which the cellular membrane is permeable. Gerard and Wallen (1929) have demonstrated that inorganic phosphate is released also in the sciatic nerves of the frog and rabbit as the result of asphyxia or tetanization. According to Gerard and Tupikow (1930) this phosphate is derived from phosphocreatin as in the case of muscle.

The increase in the inorganic phosphorus of the blood shown by Coombs, Searle and Pike (1934) during experimental convulsions in the parathyroidectomized cat, and by Weil and Liebert (1937) during epileptic attacks in man and in rabbits injected with an emulsion of Thujone, has been attributed to muscular contractions, since these increases are not observed in animals immobilized with curare.

In view of the abundance of phosphorus compounds in the central nervous system, we believe that the activity of the nervous system must be associated with a mobilization of phosphates arising in cellular metabolism; that is, that excitation of the brain must be accompanied not only by the release of potassium ions, as Cicardo and Torino (1942) and Cicardo, Torino and Fendrik (1942) have demonstrated, but also by the release of phosphate ions. The greatest concentration of these should be observed in the veins emerging from the brain, such as the superior sagittal venous sinus. Thus convulsive states should cause a mobilization of phosphorus, not only in the muscles but also in the nerve centers, indicating the most intense metabolic activity.

In order to demonstrate this release of phosphate by the brain, we proceeded to measure the plasma concentration of the total acid-soluble phosphorus in the blood collected from the superior sagittal sinus in the dog. Measurements were made before and after electrical excitation of the cerebral hemispheres by a tetanizing current or by convulsant drugs such as cardiazol or picrotoxin. Samples of blood were taken from the systemic circulation simultaneously with the samples of blood from the brain; by this means it was possible to show that the most striking increases in phosphate occurred in the blood coming from the brain.

The experiments in which the brain was excited electrically were carried out in animals in which muscular contractions were prevented by section of the spinal cord immediately below the medulla. When convulsant drugs were to be injected, the spinal cord was destroyed completely below the level of the centers

for the phrenic nerves. In similar experiments convulsions were prevented by means of curarizing agents which paralyzed the animal completely.

METHOD. The electrical excitation of the brain was carried out in dogs anesthetized with sodium ethyl barbiturate (Embotal). An induction coil was used with wire electrodes, which were placed on both hemispheres after trephine openings had been made in the cranium. In order to abolish the muscular contractions produced by electrical excitation, the spinal cord was transected immediately below the medulla, and the animal was maintained by artificial respiration through a tracheal cannula.

When the brain was stimulated with cardiazol or picrotoxin in dogs anesthetized with ether, the spinal cord was destroyed by opening the vertebral canal in the mid-dorsal region and passing a wire forward until the forelimbs were paralyzed. Diaphragmatic respiration was preserved by leaving the phrenic centers intact. The wire was also passed backward to the caudal end of the vertebral canal. This operation, which was carried out in a few minutes, produced a total paralysis except for movements of the animal's head. In some of the experiments in which the brain was stimulated either electrically or by means of convulsant drugs, muscular contractions were prevented by a crude extract of *Erythrina crista galli*, which causes complete curarization and makes artificial respiration necessary.

Samples of blood were taken simultaneously from the superior longitudinal sinus through a trephine-opening in the mid-line of the cranium and from the femoral artery or vein which had been prepared previously. The blood was drawn with heparin and was centrifuged to obtain the plasma. The flow of blood from the sinus through the opening in the cranium could be facilitated by injecting heparin into the animal to prevent the formation of clots. Samples of blood were taken before and during the electrical excitation, which lasted 2 to 5 minutes. After a rest period of 5 minutes, new blood samples were taken and the stimulation was repeated, in order to determine whether the same changes occurred as at the beginning of the experiment. When the stimulation was produced with cardiazol (pentamethylene tetrazol) injected in a dose of 0.20 to 0.30 gram, simultaneous samples were taken from the sinus and from the femoral artery before, immediately after and 10 to 15 minutes after the convulsion. When picrotoxin was injected in a dose of 5 to 10 mgm., blood samples were taken when the convulsion was at its height and 15 minutes later.

The determinations of the total acid-soluble phosphorus of the plasma were made by the method of Fiske and Subbarow.

RESULTS. Electrical excitation of the brain in anesthetized dogs or the injection of cardiazol or picrotoxin consistently produces an increase in the total acid-soluble plasma phosphorus of blood from the superior sagittal sinus, an increase which is more marked than that found in the general circulation by taking samples from the femoral artery. The local increase in phosphorus in the venous blood flowing from the brain permits one to conclude that it is in this organ that the release of phosphate occurs.

In the dogs rendered spinal by section of the cord immediately below the me-

dulla, a procedure which eliminated muscular contractions during the electrical stimulation except for those of the head and neck, the differences in the concen-

TABLE 1

● *Milligrams per cent of total acid-soluble plasma phosphorus of the superior sagittal sinus and the femoral artery, before and after electrical stimulation of the brain in spinal dogs*

EXP. NO.	BEFORE STIMULATION	AFTER 5 MIN. OF STIMULATION	15 MIN. REST		5 MIN. STIMULATION REPEATED		INCREASE
			Sinus	Artery	Sinus	Artery	
							per cent
1	2.00	2.29	3.57	3.00	4.72	3.00	+136
2	2.29	2.43	3.00	2.32	4.29	2.52	+86
3	3.57	3.57	3.57	3.57	4.29	3.57	+20
4	2.14	3.00	3.29	2.72	3.57	3.15	+66
5	2.57	3.72	4.00	3.57	5.29	4.43	+106
6	3.72	4.00	4.72	4.00	6.00	4.29	+61
7	2.72	3.00	3.00	3.00	4.00	3.57	+47
8	2.72	3.86	5.43	5.43	6.86	6.43	+152
9	2.72	3.43	3.29	3.29	3.72	3.72	+37
10	2.14	2.72	2.43	2.14	2.72	2.43	+27
Average.....	2.66	3.20	3.63	3.30	4.55	3.71	+74
Standard error.....	±0.18	±0.18	±0.29	±0.29	±0.39	±0.37	

TABLE 2

Milligrams per cent of total acid-soluble plasma phosphorus of the superior sagittal sinus and the femoral artery in curarized dogs, before and after stimulation of the brain

EXP. NO.	BEFORE STIMULATION	AFTER 5 MIN. OF STIMULATION	15 MIN. REST		5 MIN. STIMULATION REPEATED		INCREASE
			Sinus	Artery	Sinus	Artery	
							per cent
1	4.86	5.15	4.15	4.00	4.72	4.00	+14
2	4.29	4.43	3.43	3.43	3.43	3.43	+3
3	3.15	4.43	3.29	3.00	3.15	3.15	+40
4	2.86	3.86	4.29	3.86	4.29	3.86	+50
5	3.72	3.86	3.29	3.29	3.72	3.29	+13
6	2.57	3.15	2.86	2.29	3.00	2.57	+22
7	5.15	5.15	3.15	3.15	3.15	3.15	0
8	3.86	4.15	3.86	3.00	3.00	3.00	+8
9	3.29	3.86	3.29	3.29	3.29	3.29	+18
10	4.29	4.43	4.29	4.29	4.86	4.58	+13
Average.....	3.80	4.25	3.59	3.36	3.66	3.43	+18
Standard error.....	±0.27	±0.19	±0.17	±0.18	±0.22	±0.18	

trations of phosphorus ranged from +20 per cent to +152 per cent with a mean of +74 per cent in a series of 10 experiments. The increases in the general circulation were less and were probably due to phosphorus from the brain.

In curarized animals, the increases in phosphorus obtained as a result of electrical stimulation of the brain were less than those recorded in the previous circumstances, especially during the second period of stimulation 15 minutes later, when only slight, inconstant increases were observed.

The stimulation produced by cardiazol likewise caused consistent increases in the total acid-soluble phosphorus of the venous blood flowing from the brain, which in some cases amounted to 50 per cent. The blood samples taken imme-

TABLE 3

Milligrams per cent of total acid-soluble plasma phosphorus of the superior sagittal sinus and the femoral artery, before and after stimulation with cardiazol, in dogs with the spinal cord destroyed

EXP. NO.	BEFORE STIMULATION		AFTER CONVULSION		15 MIN. AFTER CONVULSION		INCREASE
	Sinus	Artery	Sinus	Artery	Sinus	Artery	
							per cent
1	8.00	8.00	8.87	8.44	7.58	7.15	+10
2	6.43	6.43	6.86	6.15	6.15	6.15	+7
3	4.72	4.72	5.43	5.15	5.00	4.86	+15
4	4.29	4.15	4.72	3.86			+10
5	3.72	3.72	4.15	3.72	4.30	3.72	+16
6	4.43	4.29	4.86	4.72	6.72	6.43	+52
7	5.29	5.29	6.15	5.43	6.86	6.15	+30
8	4.43	4.43	4.86	4.15	4.15	4.15	+10
9	2.57	2.57	2.86	2.57	2.86	2.14	+11
10	4.58	4.72	5.43	4.86			+18
11	5.29	5.29	5.72	5.43	5.43	5.43	+8
12	7.15	6.58	7.44	6.72	7.15	7.00	+4
13	6.72	6.43	7.72	6.86			+15
14	4.00	3.57	4.86	4.58	6.01	5.72	+50
15	5.15	5.15	6.58	6.01	7.15	6.58	+39
16	6.72	5.58	6.86	6.43	7.86	7.29	+17
17	3.86	3.72	4.15	3.86	4.43	4.15	+15
18	3.57	3.15	4.15	3.72	3.86	3.86	+16
19	4.86	4.86	5.15	4.58	5.00	4.72	+6
20	4.86	4.86	5.15	4.86	5.72	5.15	+18
21	3.72	3.72	4.29	4.15	4.58	4.58	+23
22	3.57	3.29	4.00	3.43	4.29	3.29	+20
Average.....	4.90	4.75	5.47	4.99	5.53	5.18	+19
Standard error.....	±0.29	±0.29	±0.30	±0.29	±0.32	±0.32	

diately after a convulsion and 15 minutes later actually showed the maximum release of phosphorus from the brain. If the curve showing the release of phosphorus by the brain is established by taking samples every 5 minutes, it is seen that this increase reaches its maximum value some time after the convulsion. If the animal passes into coma, the increase becomes progressively greater. As is shown in table 3 the increases were always more marked in the cerebral sinus than in the peripheral circulation; furthermore, since the contractions had been

eliminated by the destruction of the cord, one can be certain that the increase in phosphorus was of cerebral origin.

In those animals in which the cord had been destroyed the concentration of phosphorus before stimulation was generally found to be greater in the cerebral sinus than in the femoral arteries, and this may have been due to the activity of the brain in animals lacking muscular tone. The average of 22 experiments showed before the injection of cardiazol 4.90 mgm. per cent of total acid-soluble phosphorus in the cerebral venous sinus and 4.75 mgm. per cent in the femoral artery. The cardiazol convulsion accentuated this difference by the stimulating action of the drug on the brain. In the samples taken immediately after the convulsion an average of 5.47 mgm. per cent was found in the blood from sinus and 4.99 mgm. per cent in the blood from the femoral artery; 15 minutes later

TABLE 4

Milligrams per cent of total acid-soluble plasma phosphorus of the superior saggital sinus and the femoral artery, before and after stimulation with picrotoxin, in dogs with the spinal cord destroyed

EXP. NO.	BEFORE STIMULATION		AFTER STIMULATION		15 MIN. AFTER CONVULSION		INCREASE
	Sinus	Artery	Sinus	Artery	Sinus	Artery	
1	3.86	3.86	4.29	3.86			per cent
2	5.72	5.72	5.72	5.29	7.86	7.86	+11
3	4.58	4.15	5.29	4.72	6.15	6.15	+38
4	4.00	3.86	4.72	4.43	5.43	5.00	+34
5	4.72	4.72	5.43	5.00	5.43	5.15	+35
6	7.15	6.58	7.44	6.72	7.15	7.00	+15
7	4.15	3.86	7.00	7.00			+4
8	7.72	6.58	8.00	7.29			+69
Average.....	5.24	4.91	5.99	5.54	6.40	6.23	+4
							+26

the values were 5.53 mgm. per cent and 5.18 mgm. per cent respectively. The average increase was +19 per cent.

With picrotoxin the stimulation occurred some minutes after the injection, but the convulsions continued and frequently caused the death of the animal; hence the samples had to be taken at the height of a convulsion. It can be seen in table 4 that the concentrations of total acid-soluble phosphorus before the injection were 5.24 mgm. per cent in the sinus and 4.91 mgm. per cent in the femoral artery. These concentrations rose as a result of the action of picrotoxin to 5.99 mgm. per cent and 5.44 mgm. per cent at the beginning of the convulsion and to 6.40 mgm. per cent and 6.23 mgm. per cent 15 minutes later. The average increase was +26 per cent.

In the curarized animals the differences in concentration of total acid-soluble phosphorus of the plasma between the cerebral sinus and the femoral artery were insignificant or very slight, which shows that curarizing agents have not only a

peripheral but also a central action. This central effect of curarizing agents would explain the lack of increase or very slight increase in phosphorus resulting from the action of cardiazol or picrotoxin. As the curarization of these animals was prolonged, the concentrations of acid-soluble phosphorus continued to decrease.

DISCUSSION. The release of phosphate by the brain of the dog as the result of electrical stimulation or the injection of convulsant drugs such as cardiazol or picrotoxin is demonstrated in our experiments, since the increase in phosphate was observed in the superior sagittal sinus in animals in which, as a result of section or destruction of the spinal cord, muscular contractions were absent except for those of the head and neck. Furthermore, the concentrations are always greater in the venous blood flowing from the brain than in the peripheral

TABLE 5

Milligrams per cent of total acid-soluble plasma phosphorus of the superior sagittal sinus and the femoral artery in curarized dogs injected with cardiazol or picrotoxin

EXP. NO.	BEFORE INJECTION		AFTER INJECTION		15 MIN. AFTER PREVIOUS SAMPLE		INCREASE
	Sinus	Artery	Sinus	Artery	Sinus	Artery	
1	5.15	5.15	5.29	5.15	3.29	3.15	+2
2	2.57	2.29	2.86	2.86	1.43	1.43	+12
3	3.57	3.57	4.15	4.15	4.29	4.58	+20
4	6.00	6.00	6.43	6.00	6.43	6.43	+7
5	5.29	5.29	5.29	5.29	4.43	4.43	
6	3.29	3.29	3.57	3.57	2.57	2.43	+9
7	3.57	3.57	4.15	4.15	4.15	4.15	+16
8	3.29	3.29	3.29	3.29	3.15	3.15	
9	5.00	4.58	4.43	4.43	3.57	3.57	+11
10	3.43	3.15	3.43	3.29	3.00	3.00	
Average.....	4.16	4.02	4.29	4.22	3.63	3.63	+5.5

circulation. If the phosphorus set free were not of cerebral origin, the concentration in the peripheral blood should be equal to or greater than that in the cerebral veins.

The fact that in curarized animals the increases are not observed or are only slight does not mean that the notion of cerebral origin should be rejected, but that curarizing substances should be thought of as having not only a peripheral but also a central action, as has already been pointed out. In fact, even before the injection of convulsant drugs the concentrations of total acid-soluble plasma phosphorus in curarized dogs are found to be similar in the cerebral sinus and in the femoral artery, a situation which is not observed in animals immobilized by destruction of the cord; in these a greater concentration is found in the cerebral veins indicating the activity of the brain. Cicardo and Torino (1942) have demonstrated a similar phenomenon in the release of potassium by the curarized

brain upon stimulation which behaves with respect to this ion as it does with respect to phosphorus.

The release of inorganic phosphorus produced by electrical stimulation or by convulsant drugs is an indication of cellular metabolic activity, and as in the case of the muscle, according to the studies of Embden and Adler (1922) and of Eggleton (1929), and in nerve, according to Gerard and Wallen (1929), might be due to the hydrolysis of phosphocreatin or of adenosine triphosphate.

While the release of potassium by the brain, demonstrated by Cicardo and Torino (1942) during electrical stimulation and by Cicardo, Torino and K. Fendrik (1942) with convulsant drugs, occurs at the same time as the beginning of cerebral excitation, in the case of phosphorus the release is found to be slower and to reach a maximum some time after the convulsion has occurred. This phenomenon could be explained by the greater speed of diffusion of potassium or by considering the release of phosphates a slower metabolic process.

CONCLUSIONS

1. The stimulation of the brain of the dog by a tetanizing electric current or by cardiazol or picrotoxin causes a release of phosphorus which is indicated by the increase of the total acid-soluble plasma phosphorus of the blood collected from the superior longitudinal venous sinus.

2. The cerebral origin of this phosphorus is demonstrated by the fact that muscular contractions were eliminated by the section of the cord below the medulla, or by destruction of the cord when drugs were to be injected, and also by the fact that samples taken simultaneously from the femoral artery or vein do not show similar increases.

3. The release of phosphorus increases after the convulsion is over and persists as long as 15 minutes, depending on the intensity of the convulsion.

4. In animals immobilized by destruction of the cord, even before the convulsant drug is injected, the concentration of phosphorus is found to be somewhat greater in the cerebral sinus than in the femoral artery, probably because of the continuing activity of the brain and the absence of muscular tone.

5. The difference mentioned above is not observed in curarized animals, perhaps because of the central action of the curarizing substance, a fact which would cause electrical or chemical stimulation in these animals to produce little or no release of phosphorus. The plasma phosphorus decreases progressively in curarized animals.

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THE EFFECT OF DOSE AND NUTRITIVE STATE ON THE RENOTROPHIC AND ANDROGENIC ACTIVITIES OF VARIOUS STEROIDS¹

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In a previous report from this laboratory (1) it was demonstrated that the renotrophic and androgenic properties of various steroids were influenced by the chemical structure of the material administered. Androstenediol-3 α ,17 α and its 17-methyl derivative demonstrated a preferential renotrophic effect. These observations have been confirmed (2). Furthermore, a study of the relative renotrophic and androgenic properties of the steroids suggested that in some cases the rate of absorption of the steroids was too great and in others too small to provide the most efficacious amount of the compounds. Therefore, it seemed advisable to extend the previous study (1) so that renotrophic and androgenic values at various dose levels of the steroids might be compared.

METHODS. *Pellets.* The steroids² were made into cylindrical pellets of 14 ± 1 mgm., diameter 2.7 mm. and length 2.7 ± 1 mm., by means of a hand-press designed in this laboratory.

In order to decrease the rate of absorption of the more soluble steroids they were thoroughly mixed with cholesterol in various proportions (3, 4) by grinding the two components in an agate mortar. An increase in the amount of steroid absorbed was obtained by implanting two or more pellets of the pure steroid.

*Animals.*³ Mice of the highly inbred Murray-Little dba strain were castrated under ether anesthesia at 16.0 to 19.5 grams body weight. They were kept in the animal house and fed Purina Fox Chow checkers.

Duration of experiments. The steroid pellets were implanted thirty days after castration and were allowed to remain *in situ* for thirty days.

Autopsy. The mice were fasted 24 hours before autopsy and were killed by decapitation. The organs were immediately removed and weighed on a Roller-Smith torsion balance. The pellets were removed, washed in distilled water dried in a desiccator over calcium chloride and reweighed to determine the amount absorbed. The amount of steroid hormone absorbed from the pellets composed of mixtures with cholesterol was obtained by multiplying the total amount absorbed from the pellet by the per cent of steroid present in the original

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²The pure steroids were provided by Ciba Pharmaceutical Products Inc. through the courtesy of Drs. C. R. Scholz and Ernst Oppenheimer. Many of the compounds were prepared by Doctor Scholz specially for this and related studies.

³The mice were provided by Dr. S. G. Warner, Biological Station, Springville, N. Y.

pellet. Although cholesterol is not absorbed when implanted subcutaneously (3, 4), a pellet composed of a mixture of cholesterol and a soluble steroid loses weight in proportion to the materials present⁴ (4).

RESULTS. *The effects of castration.* The values for the normal and the castrated mice are the same as obtained previously (1).

Renotrophic activity. The more active steroids bring about a very rapid and similar increase in kidney size. Once the maximum rate of increase has been attained, further increase in dose produces only a slight but continuous increase in size of the organ. These effects are illustrated graphically for testosterone in figure 1.

Androgenic activity. Testosterone; testosterone propionate; 17-methyl testosterone and androstanol-17 α , one-3 produce as rapid and great an effect on the

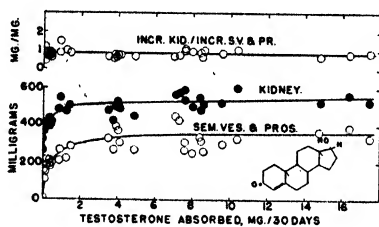


Fig. 1

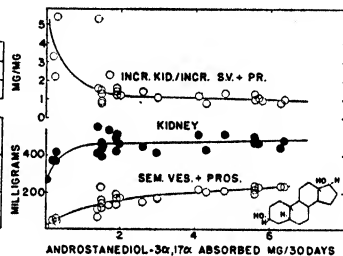


Fig. 2

Fig. 1. A comparison of the renotrophic and androgenic activities of testosterone at different doses. The values for the castrated control mice represent the averages of 8 mice (cf. table 1); the values for testosterone are individual determinations. The values for the normal mice are: Kidneys 414, Seminal Vesicles and Prostates 222 and Incr. Kid./Incr. S. V. & Pr. = 0.74.

Fig. 2. A comparison of the renotrophic and androgenic activities of androstanediol-3 α , 17 α .

seminal vesicles and prostates as on the kidneys but the diols produce a much slower effect (cf. fig. 2) especially in the lower, more efficacious doses (fig. 3).

Renotrophic-androgenic ratio. When the increased kidney weight is divided by the increased seminal vesicles and prostate weight, testosterone and its derivatives and androstanol 17 α , one-3 produce a ratio slightly less than one which is not affected by dose (table 1, cf. also fig. 1). The diols (table 1, cf. also fig. 2), on the other hand, produce a ratio much greater than one which gradually decreases with increase in dose.

Renotrophic efficacy. The renotrophic efficacies of testosterone; testosterone propionate; 17-methyl testosterone; androstanol-17 α , one-3; 17-methyl androstanediol-3 α , 17 α and androstanediol-3 α , 17 α not only are the same when

⁴The cholesterol and steroid hormone ratio in the recovered pellets of this study have been determined and are in the same ratio as present originally. The cholesterol apparently sloughs from the pellet as the steroid is absorbed.—A more complete discussion of absorption from steroid pellets will be presented later.

The effect of the amount of steroid absorbed on the weight of the kidney, seminal vesicles and prostates and the thymus of the castrated mouse (dba strain)

TREATMENT	NO. OF MICE	PEL-LETS§ IMPL'T'D	STEROID ABS'D.	INCREASED WEIGHT				INCR'D. KID WGT.	THY-MUS
				Kidneys		Sem. Ves. & Pros.			
				mgm./30d.	%	mgm./mM × 288¶	%	mgm./mM × 288¶	
Normal mice.....	12			57		1920		0.74	21
Cholesterol.....	8	1 or 2	0.0**	(265)††		(11)††			32
Testosterone.....	4	1:4	0.14	47	900	1310	1030	0.87	20
Testosterone.....	4	1:2	0.37	57	416	1720	511	0.82	7
Testosterone.....	4	1:1	1.15	91	210	2210	211	1.00	7
Testosterone.....	7	2:1	4.06	77	47	2730	69	0.68	3
Testosterone.....	3	2	16.2	104	17	3220	22	0.79	4
Testosterone propionate*	4	1:2	0.20	49	806	1530	1050	0.77	23
Testosterone propionate*	4	1:1	0.88	73	275	2090	324	0.85	14
Testosterone propionate*	5	2:1	2.33	85	121	2340	137	0.88	6
Testosterone propionate*	5	2	9.40	106	37	2630	38	0.97	3
17-Methyl testosterone†.....	4	1:4	0.14	60	1142	1160	962	1.19	16
17-Methyl testosterone†.....	4	1:2	0.57	82	298	1970	402	0.74	9
17-Methyl testosterone†.....	4	1:1	1.30	91	164	2200	195	0.84	7
17-Methyl testosterone†.....	6	2:1	4.26	99	60	2390	65	0.93	6
17-Methyl testosterone†.....	4	2	14.40	111	19	2680	22	0.89	3
Androstanol-17 α , one-3.....	5	1:1	0.65	60	248	1680	285	0.87	9
Androstanol-17 α , one-3.....	2	2	6.40	95	40	2580	44	0.90	6
Androstanol-17 α , one-3.....	4	3	7.10	91	36	2640	41	0.84	6
Androstanediol-3 α , 17 α	3	1:1	0.25	45	474	330	142	3.33	22
Androstanediol-3 α , 17 α	4	2	3.80	78	54	1550	44	1.22	10
Androstanediol-3 α , 17 α	4	3	5.50	75	36	1840	37	0.99	4
Androstanediol-3 α , 17 α	3	4	5.90	76	34	2000	37	0.92	5
17-Methyl androstanediol-3 α , 17 α	4	1:1	0.60	73	344	860	165	2.09	13
17-Methyl androstanediol-3 α , 17 α	5	2	3.20	95	84	1790	65	1.28	8
17-Methyl androstanediol-3 α , 17 α	6	3	5.50	70††	36	1740	37	.97	6
17-Methyl androstanediol-3 α , 17 α	5	4	6.90	92	37	1980	33	1.12	6
17-Methyl androstanediol-3 β , 17 α	7	3	1.80	60	92	290	19	4.90	18
17-Methyl Δ^5 -androstenediol-3 β , 17 α	4	4	2.40	32	37	400	15	2.47	30
Δ^4 -Androstenedione-3, 17.....	2	2	19.70	60	8	1950	11	0.73	10
Testosterone benzoate.....	2	5	0.9	28	97	670	99	0.98	40
17-Ethynyl testosterone†.....	2	3	1.8	-2	0	50	3		34
Androstanediol-3 β , 17 α	4	4	2.0	27	30	0	0		34

* Perandren.

† Metandren.

‡ Pregnenynolone, Lutocylol.

§ The figures in this column indicate the number of pellets or the steroid:cholesterol composition of the pellet implanted in each mouse.

|| Average changes from average values of castrated control, cholesterol treated, mice.

¶ The millimoles of each steroid have been multiplied by the molecular weight, 288 of testosterone.

** Average deviation, ± 0.15 .

†† These values are in milligrams.

‡‡ The inability to demonstrate a maximum renotropic effect in these mice was due to the fact that these animals were slightly undernourished as indicated by their body weights (cf. table 3).

compared on a mole basis but also show the same rapid decrease in efficacy with increase in dosage (fig. 4). The other compounds with the possible exception of methyl androstenediol- $3\beta,17\alpha$, which is only slightly less active than the above steroids, show varying degrees of lower efficacy.

Androgenic efficacy. Testosterone; testosterone propionate; 17-methyl testosterone and androstanol- 17α , one-3 possess the same androgenic activity when compared on weight equivalent basis (fig. 3). The diols on the other hand are much less efficacious than the above steroids at the lower levels but have the same activity at the higher dose levels at which all of the compounds have a relatively low efficacy (fig. 3). A further decrease and even an inhibition of androgenic activity is obtained by acetylation or the substitution of a 3β -OH group for the 3α -OH group.

Less active steroids. When testosterone benzoate; 17-ethynyl testosterone; androstenediol- $3\beta,17\alpha$; 17-methyl androstenediol- $3\beta,17\alpha$ and 17-methyl an-

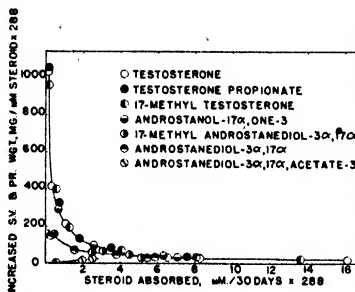


Fig. 3

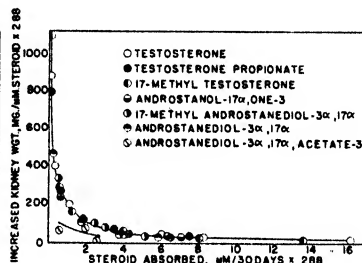


Fig. 4

Fig. 3. The renotropic efficacy of the more active steroids at different doses.

Fig. 4. The androgenic efficacy of the more active steroids at different doses.

drostenediol- $3\beta,17\alpha$ were studied by the implantation of a single pellet of the respective compounds (1), they showed no or slight activity. It seemed that the low degree of activity might be due in some measure to the very low rate of absorption of these compounds. Therefore, these compounds were studied by implanting several pellets of the steroids in order to materially increase the amount of steroid absorbed. As a result all of these compounds with the exception of 17-ethynyl testosterone produce a physiological response. The efficacies of these compounds, however, are not as great as those of the more active compounds. The 17-methyl group enhances the activities of androstenediol- $3\beta,17\alpha$ (cf. 1).

The inability of Δ^4 -androstenedione- $3,17$ to attain as great an absolute effect as the most active compounds even though relatively tremendous amounts of this compound were made available to the organism indicates that this steroid possesses only moderate physiological activity. This is of special interest since Δ^4 -androstenedione- $3,17$ is the chief metabolic product isolated after the incubation of testosterone with rabbit liver slices (5). Furthermore, testosterone

is the chief metabolite when Δ^4 -androstenedione 3,17 is used as the substrate (6). Thus the body seems to set up an equilibrium between these two steroids.

Nutritive state. At one time in the course of this investigation the mice did not obtain adequate care due to technical difficulties, consequently they were in a state of undernutrition during most of the experimental period. As a result there was a marked decrease in the weight of the kidneys and seminal vesicles and prostates of the normal mice (cf. 7) but only a relatively small decrease in these organs of the castrated mice (table 2). Furthermore, the ability of the underfed castrated mice to respond to androgenic stimulation was not impaired but was to renotrophic stimulation (table 3).

DISCUSSION. The ability of testosterone, testosterone propionate; 17-methyl testosterone, androstanol-17 α , one-3; androstanediol-3 α ,17 α ; and 17-methyl androstanediol-3 α ,17 α to produce similar renotrophic effects but not similar androgenic effects when administered at equivalent dose levels emphasizes the importance of dose and chemical structure to physiological activity. Further-

TABLE 2

Effect of the nutritive state on the body, kidney and seminal vesicles and prostate weights

NUTRITIVE STATE	MICE	NO. OF MICE	FASTING BODY WGT.	SEM. VES. +PROS.	KIDNEY
			grams	mgm.	mgm.
Normal.....	Normal	12	22.5	222	414
Undernutrition.....	Normal	3	13.9	69	222
Normal.....	Castrated	8	20.7	11	265
Undernutrition.....	Castrated	6	16.1	8	235

more, those compounds that have similar effects on both the kidney and seminal vesicles and prostates demonstrate differences in other respects. Methyl testosterone is able to more rapidly increase the arginase activity of the kidney of the mouse (8) and the creatine production and excretion by man (9, 10) than testosterone or testosterone propionate. A similar difference is noted between 17-methyl androstanediol-3 α ,17 α and androstanediol-3 α ,17 α . Thus, no one criterion is adequate for a comparison of the physiological properties of the steroid hormones. One compound may be particularly efficacious for one physiological effect but have a similar, decreased or no effect on other processes.

There is as yet no evidence to indicate that the steroid hormones are absorbed and produce their effects in the body as such. On the contrary it is more likely that the administration of one steroid results in its conversion by the body to a number of substances which bring about the manifold effects attributed to each compound. Thus the physiological effects of a compound would depend not only upon itself but also on the other compounds to which it was metabolised by the body. The rabbit liver is able to convert testosterone to Δ^4 -androstenedione-3,17, cis-testosterone and many other compounds (5). Furthermore, it can convert Δ^4 -androstenedione-3,17 to testosterone, cis-testosterone and other

steroids (6). The liver probably is not the only organ in the body that can metabolise the steroids (11). It would seem, therefore, that the physiological effect of a compound would depend upon the state of equilibrium among the many steroid compounds and this equilibrium in turn would depend on the particular compound made available to the body. It may be assumed then that

TABLE 3

Effect of the nutritive state on the response of the kidney and the seminal vesicles and prostates to steroid stimulation

NUTRITIVE STATE	TREATMENT	NO. OF MICE	FASTING BODY WEIGHT	STEROID ABSORBED	INCREASED WGT.†	
					S. V. +Pr.	Kidney
			grams	mgm./30 days	mgm.	mgm.
Normal.....	Testosterone	4	20.8	0.37	189	154
Undernutrition....	Testosterone	1	16.4	0.50	188	87
Normal.....	Testosterone	4	22.4	1.15	243	242
Undernutrition....	Testosterone	1	17.2	1.20	272	128
Normal.....	Testosterone	7	20.6	4.34	300	203
Undernutrition....	Testosterone	5	17.1	4.40	214	122
Normal.....	Testosterone	3	22.4	16.2	354	278
Undernutrition....	Testosterone	1	18.7	17.8	313	196
Normal.....	Testosterone* propionate	5	21.6	2.33	257	227
Undernutrition....	Testosterone* propionate	4	16.3	2.67	207	97
Normal.....	17-Methyl-testosterone†.....	4	23.3	0.14	128	152
Undernutrition....	17-Methyl-testosterone†	1	18.0	0.12	122	64
Normal.....	17-Methyl-testosterone†	6	22.8	4.26	263	244
Undernutrition....	17-Methyl-testosterone†	3	17.3	3.54	232	144
Normal.....	17-Methyl androstanediol- 3 α ,17 α	5	22.5	3.2	197	254
Undernutrition....	17-Methyl androstanediol 3 α ,17 α	2	16.0	3.8	168	67
Normal.....	Androstanediol-3 α ,17 α	3	22.1	0.25	36	120
Undernutrition....	Androstanediol-3 α ,17 α	1	18.9	0.30	36	17
Normal.....	Androstanediol-3 α ,17 α , Ace tate-3	12	21.9	2.5	48	151
Undernutrition....	Androstanediol-3 α ,17 α , Ace- tate-3	4	14.7	3.2	45	60

* Perandren.

† Metandren.

‡ Change from values of castrated mice in a normal nutritive state.

compounds which have equal physiological effects are converted to the same amount and types of steroids.

The diols are of particular interest because of their low androgenic but high renotrophic activity. Thus, if the renotrophic response is any indication of their effect on metabolic processes, then these compounds would be of advantage in metabolic studies where sexual effects are undesirable.

The androgenic property of the steroid molecule seems to be associated in

great part but not entirely (1) with the nature of the grouping at the 3 position in the molecule. The renotrophic property of the molecule is not but the androgenic is affected when an α -hydroxyl group is introduced in this position. Furthermore, acetylation of this group or conversion of it to its β stereoisomer results in a further decrease in relative androgenic activity (also cf. 1).

The great decrease in efficacy of the steroids with increase in dosage was not too surprising. It was, however, of interest to note that the amount of the most active steroids necessary to restore the kidney or seminal vesicles and prostates to normal size is very small. For example, the mouse seems to require only about 15 $\mu\text{g.}/\text{day}$ of testosterone or its equivalent to restore its kidney size to normal in a 30-day period. This is equivalent to 3 $\text{mgm.}/\text{m}^2$ body surface/day or about 0.67 $\text{mgm.}/\text{kgm.}/\text{day}$ for a 22.5 gram mouse.

The rapid attainment of a maximal response of the kidney to steroid stimulation does not mean that a similar effect has been attained for all the other properties of these compounds. The arginase activity of the kidney continues to increase with increase in dose of steroid long after the kidney has attained its maximum response (13).

The simultaneous and similar loss in weight by the kidneys and seminal vesicles and prostates of the normal animals during undernutrition is very likely due to the loss of endocrine stimulus through the now well known phenomenon of inanition hypophysectomy. These same organs in the castrated animals lose relatively small amounts of tissue mass because they have already regressed to a basal level as a result of surgical removal of their endocrine stimulus. When, however, the stimulus for growth of both of these organs is restored by administration of steroid pellets, there is a marked difference in the response of the two organs in the underfed mice. The seminal vesicles and prostates increase in size to as great an extent as in the well-fed animals. The kidneys, on the other hand, show a very greatly decreased response. Thus the increase in size of these two organs under steroid stimulation is for entirely different purposes. The accessory sex organs increase for their own particular and special functions. The kidney, on the other hand, increases in size to store reserve protein which it probably synthesizes under the stimulus of the steroid hormones. During inanition it gives up the newly fabricated material for other more vital needs. Thus the kidney may be considered as an intermediary in some phases of protein fabrication (cf. 14).

The effect of the nutritive state of the animal on the renotrophic and androgenic properties of the steroids emphasizes the utmost importance for exactly controlled conditions in studying these effects.

SUMMARY

When testosterone, testosterone propionate, 17-methyl testosterone, androstano-17 α , one-3; androstanediol-3 α , 17 α and 17-methyl androstanediol-3 α , 17 α pellets are implanted subcutaneously in mice they produce the same renotrophic effect per mole equivalent. The first four steroids also produced the same androgenic effect but the diols were much less effective in this respect.

Acetylation or substitution of a 3β -hydroxyl group for the 3α -hydroxyl group resulted in further decrease in androgenic potency. The renotrophic and androgenic efficacies of all of the compounds were rapidly and markedly decreased with increase in dose. The amount of the most active compounds necessary to restore the kidneys and seminal vesicles and prostates to normal size in the castrated mouse was very small; 15 μ g. or less per day were required over a 30-day period.

During undernutrition the castrated mouse loses relatively smaller amounts of kidney tissue than the normal mouse. The administration of active steroids to these animals produces as great an accessory sex organ response but a much lower kidney response than that in the well-fed castrated mouse.

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THE METABOLISM OF ACETATE BY THE COMPLETELY ISOLATED MAMMALIAN HEART INVESTIGATED WITH CARBOXYL-LABELED ACETATE

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Barcroft, McAnally and Phillipson (1) have shown that added acetate disappears from a saline fluid perfusing the isolated rabbit heart. Toennissen and Brinkmann have reported similar findings using skeletal muscle of the rabbit perfused with diluted blood (2). The experiments reported in the present communication demonstrate that when acetate labeled with heavy carbon in the carboxyl group is administered to the completely isolated, working, cat heart, the isotope appears in the respiratory CO_2 .

METHODS. The isolated heart preparations were set up essentially as previously described (3), except for CO_2 absorption, which was carried out by passing the respiratory gas mixture through a dispersion filter in a bottle containing 2N carbonate-free NaOH. At intervals, a fresh bottle was inserted in the circuit. Total CO_2 production was measured by analysis of the alkali in the Van Slyke manometric apparatus, and the C^{13} content of the CO_2 was determined in the Nier mass spectrometer (4). Acetic acid containing 5.26 atoms per cent excess C^{13} in the carboxyl carbon was synthesized from methyl bromide by the Grignard reaction, and was administered as the sodium salt, dissolved in water. Enough dilute HCl was added to the solution to bring it just to the acid side of phenolphthalein. The acetate was injected in two equal doses of 5 cc. Each dose was given slowly over a thirty minute period, the first injection beginning at 0 time, and the second, at the midway mark.

A control experiment was done in which blood containing labeled acetate was pumped through the system, in the absence of the heart. CO_2 was collected and analyzed for its C^{13} content.

RESULTS AND DISCUSSION. The principal results are presented in table 1. In two experiments (nos. 1 and 2) approximately $5\frac{1}{2}$ millimoles of labeled sodium acetate were injected into the blood perfusing the isolated heart. It is evident that an appreciable excess of C^{13} appeared in the respiratory CO_2 in each collection period. In the control experiment (no. 3) carried out in the same way, except that the heart was omitted, the collected CO_2 showed a normal isotope

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² Collaboration of the Department of Physiology of the University of Minnesota in these experiments was extended at the kind invitation of the Agricultural Research Council, London.

content. From these observations it is clear that the heart is necessary for the conversion of acetate to CO_2 .

The per cent of the administered isotope recovered in the respiratory CO_2 is calculated as follows:

$$\frac{\text{mM respiratory CO}_2 \times \text{atoms } \% \text{ excess C}^{13} \text{ in respiratory CO}_2}{\text{mM administered acetate} \times \text{atoms } \% \text{ excess C}^{13} \text{ in carboxyl carbon}} \times 100 =$$

per cent of administered C^{13} recovered in the respiratory CO_2 . This was found to be 17.95 per cent and 5.85 per cent in experiments 1 and 2 respectively. If we

TABLE 1

EXPT. NO.	HEART WEIGHT	BLOOD VOLUME	ACETATE* ADMINISTERED	RESPIRATORY CO_2				ADMINISTERED ACETATE UNDERGOING NET CONVERSION TO CO_2	PER CENT OF TOTAL RESPIRATORY CO_2 DERIVED FROM ADMINISTERED ACETATE†
				Individual collection periods	mM collected	Atoms, per cent excess C^{13}	Per cent of administered C^{13} recovered		
1	grams	cc.	mM.	minutes				1.35	31.5
	13	125	5.6	50.5	1.79	0.32	1.98		
				72.5	2.11	0.66	4.79		
				80.0	2.52	0.78	6.80		
					1.88‡	0.78	4.38		
							17.95		
2	7.7	90	5.4	58	0.99	0.28	1.00	0.92	19.5
				61	1.46	0.48	2.43		
				40	0.80	0.51	1.43		
					0.65‡	0.51	0.99		
							5.85		
3	No heart	80	3.85	205	0.95	0.00	0.00		

* The acetate contained 5.26 atoms per cent excess C^{13} in the carboxyl carbon.

† $\text{mM Acetate administered} \times \% \text{ administered acetate converted to CO}_2 \times 2 \div \text{total mM of respiratory CO}_2 \text{ collected} \times 100$.

‡ Residual CO_2 in blood and ventilating gas at the end of the experiment. The C^{13} in this CO_2 was assumed to be the same as that found in the last collection period.

assume that CO_2 arises at an equal rate from the α and carboxyl carbons, then the fraction of administered C^{13} recovered in the respiratory CO_2 also represents the fraction of the administered acetate undergoing net conversion to CO_2 . This assumption gains support from the fact that in preliminary experiments in the rat fed carboxyl as well as carboxyl and α labeled acetate, CO_2 appears to arise at an equal rate from both carbons of the acetate molecule (5). Thus it is probable that about 18 per cent and 6 per cent of the administered acetate has undergone net conversion to CO_2 in experiments 1 and 2, respectively. The difference noted in the two experiments is partly due to the difference in the size

of the two hearts and in the duration of the two experiments. When reduced to unit weight of heart muscle, and unit time, the discrepancy is not so marked, 1.35 and 0.92 mgm./gm. of heart/hour having undergone conversion to CO_2 . Barcroft, McAnally and Phillipson (1), found acetate disappeared from the Locke-perfused rabbit heart at rates as high as 8.9 mgm./gm. of heart/hour. Certain differences between the two sets of experiments, however, make the comparison of questionable meaning. Acetate was the only extracardiac substrate in the rabbit heart experiments, while blood was used in the present work, as well as the heart from a different species. In addition, disappearance, rather than conversion to CO_2 , was measured in the former studies.

From the values for net conversion of administered acetate to CO_2 , as calculated above, the per cent of the total respiratory CO_2 derived from administered acetate is seen to be 31.5 and 19.5 per cent for experiments 1 and 2 respectively.

An effort was made to gain a more complete picture of the fate of the injected acetate. In experiment 1 the acetic acid recovered from the blood, and checked for purity by the partition coefficient (6), was analyzed for its C^{13} content, and 3.66 atoms per cent C^{13} was found in the whole molecule as compared to the initial value of 3.72. The significance of this difference at such C^{13} concentrations is considered to be questionable. Had administered acetate been exchanging with some intermediate, or had it been mixing with non-isotopic acetate of endogenous origin, the concentration of C^{13} in acetate recovered at the close of an experiment should have been lower than in the administered compound. However, the failure to find an analytically significant decrease in the isotope concentration of the recovered acetate does not exclude the occurrence of a metabolically significant degree of such exchange or dilution. The resulting isotope dilution may have been masked by the relatively large amounts of acetate given. For example, had acetate been arising from endogenous sources in the heart at the same rate as that calculated from isotopic data for the whole rat (15–20 mM/100 gram of rat/day) by Bloch and Rittenberg (7), the expected dilution of the administered isotope would be in the neighborhood of that found, a dilution that could have been caused by the presence of a small amount of impurity not detectable by the partition coefficient.

The presence of acetone bodies in the blood was tested for (8) but none was found.

SUMMARY

Acetic acid, labeled with the mass isotope of carbon, C^{13} , in the carboxyl position, when administered as the sodium salt to the completely isolated, working mammalian heart, is readily converted to CO_2 as evidenced by the appearance of C^{13} in the respiratory CO_2 .

We wish to express our thanks to the staff in the Physics Department of the University of Minnesota, and especially to Dr. A. O. Nier, for their generous co-operation in this investigation; to the Misses Ann Utter and Carol Carlson; and to Mr. Wayne Adams, who rendered valuable technical assistance.

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PROVISUAL RED AND VISUAL RED¹

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It is an old observation that the retina in the cold first turns orange before it bleaches to a yellow color. Lythgoe (1) noted that visual purple behaved in the same manner and called the substance which possessed the orange color, transient orange. He observed that the absorption spectra of solutions of bleached visual purple containing the pigment differed from that of other retinal pigments but he made no attempt to isolate the substance. Evidently visual purple forms an intermediate orange colored pigment which may be a precursor of visual yellow (retinene).

There is also reason to believe that visual purple is not simple conjugation of visual yellow and protein. Visual purple has the properties of a large molecule with a molecular weight estimated to be from 26,500 to 810,000 (2,3,4). Purified bovine visual purple contains from 35 to 50 per cent lipid and the remaining percentage is protein. On the basis of the lowest estimation of molecular weight of visual purple the lipid fraction has a much larger weight than that of visual yellow if visual yellow is a simple or esterified carotenoid. Furthermore the initial yield of colored lipids obtained from visual purple by any procedure so far used in this laboratory is less than one per cent. It is apparent that the lipid fraction is a large complex group carrying a small chromophoric group.

With the object of determining the nature of the lipid fraction of visual purple the isolation of the intermediate precursor of visual yellow was attempted. Since the same substance was derived from a solution of visual purple, the whole retina was used as a convenient source of material.

EXPERIMENTAL. *Preparation of provisual red.* The procedure which was used on a typical batch of retinas is described. The retinas without pigment epithelium were removed from 10,000 bovine eyes on the killing floor of the slaughter house. They weighed 13,400 grams and contained 11.84 per cent solids. The fresh retinas were at once repeatedly extracted with portions of 40 L. of ice cold acetone until free of soluble pigment. The residue was discarded. The acetone solution was diluted with water to make a 70 per cent solution and it was then exhaustively extracted with 6 L. of cold petroleum ether (b.p. 30° to 60°). The petroleum ether was evaporated under reduced pressure below 45° C. The lipid residue, marked A, weighing 64 grams was extracted with acetone leaving about 20 grams of insoluble lipid. Water was added to make 500 cc. of a 70 per cent solution of acetone. A few grams of crystalline sterol which precipitated were removed. The acetone solution was extracted with 300 cc. of petroleum ether

¹ This investigation has been supported by a grant from the John and Mary Markle Foundation.

three times. About 0.5 gram of oil remained in the acetone solution. After vacuum distillation of petroleum ether, the residue was redissolved in 20 cc. petroleum ether and passed over a column of Alorco A brand of aluminum oxide of 120 mesh. The deep red band formed 3 cm. from the top of the column was removed and elutriated with a solution of 5 per cent ethanol in petroleum ether.

The sterids were removed by adding a solution of digitonin to red lipid solution. The precipitate of digitonides was discarded. After evaporation of the solvent the excess of digitonin was removed by solution of the pigment in ether. The pigment was transferred to petroleum ether and passed over a column of Brockmann's aluminum oxide. The red layer was separated and elutriated. The solution was evaporated under a vacuum. The red lipid weighed 342 mgm., a yield of 0.00255 per cent of the fresh or 0.0215 per cent of the dry retina.

Many methods of treatment were tried unsuccessfully. Methyl and ethyl alcohol in place of acetone gave small yields of red pigment. Isopropyl and tertiary butyl alcohol held many other substances in solution which made purification difficult and doubtful. Other materials for chromatographic absorption such as Lloyd's reagent, sugar, calcium sulfate, carbonate, oxide, hydroxide, phosphate, magnesium oxide, carbonate, zinc phosphate, permutit and bleached fuller's earth were found to absorb poorly or to cause oxidation. Unless the work was done rapidly or large quantities were employed, it was necessary to use nitrogen to displace air over all solutions or solids containing colored lipids. Low illumination was only required when working with retinas and the first acetone solutions. Red safety photographic lights and ice cold solutions were preferable.

Properties. The red viscous amorphous lipid, called provisual red, was soluble in methyl and ethyl alcohol, acetone, ether, petroleum ether, chloroform, carbon tetrachloride and glacial acetic acid and was insoluble in water and in solutions of sodium hydroxide. No method was found to induce crystallization.

Anhydrous antimony trichloride in chloroform with provisual red gave a green blue color which was fairly stable for over an hour. The absorption spectra of the initial color, sea green, showed a maximum at 620 $m\mu$ (610-630 $m\mu$). At the end of 6 hours the color was red.

Girard T reagent reacted with provisual red but the lipid could not be recovered without some decomposition.

Absorption spectra. The absorption spectra of provisual red in absolute alcohol began at 540 $m\mu$ and was maximal at 440 $m\mu$ and 330 $m\mu$. In n-heptane it was suppressed about 50 per cent and the maximum was shifted 20 $m\mu$ toward the ultra-violet.

Analysis. The lipid was found to have 79.06 per cent carbon, 10.55 per cent hydrogen and 0.59 per cent nitrogen. One sample from another batch gave 79.60 per cent carbon, 10.36 per cent hydrogen, 0.53 per cent nitrogen and 0.18 per cent sulfur. No phosphorus was present in either sample.

Hydrogenation in absolute alcohol with PtO_2 as a catalyst resulted in absorption of 1.44 per cent hydrogen. The crude hydroprovisual red recrystallized from alcohol gave a m.p. 35-37 C. uncorrected.

The values obtained by the use of camphor* and cyclopentadecanone in the Rast method for determination of molecular weight were low and unsatisfactory.

In a 50 per cent alcoholic solution of 0.0463 N NaOH, 104 mgm. of provisual red showed a saponification equivalent of 990.4 which was the maximum value reached at the end of seventy-two hours. The titration value agreed well with that of the total fatty acids isolated after hydrolysis.

After alkaline hydrolysis of 312 mgm. of provisual red, 174 mgm. of visual red were shaken out of the alkaline solution with petroleum ether. The solution was acidified with hydrochloric acid and 70 mgm. of fatty acid were removed by extraction with petroleum ether, and 14 mgm. of a petroleum ether insoluble fatty acid was extracted with ether. By differences, 54 mgm. were water soluble substances. Another sample of 663 mgm. of provisual red after hydrolysis gave 159 mgm. of visual red, 354 mgm. of petroleum ether soluble fatty acid, 71 mgm. of ether soluble acid and 79 mgm. of water soluble substances. Although the yield of visual red varied, the ratio of fatty acid and water soluble substances remained fairly constant in the above and other samples. The amount of visual red in provisual red usually varied inversely with the speed of preparation of the provisual red. Apparently the ester was easily hydrolyzed.

The petroleum ether soluble acid absorbed 0.803 per cent hydrogen. After recrystallization of the hydrogenated acid from alcohol it melted at 69–70° C. uncorrected. Mixed with stearic acid the m.p. was not changed. The molecular weight from the titration value was 274. Analysis was in accord with that of stearic acid. It is probable that the acid was oleic acid.

The ether soluble acid was pale yellow in color and was soluble in 50 per cent aqueous ethyl alcohol. It absorbed 1.28 per cent hydrogen. The neutral equivalent of the hydrogenated acid was 291. The p-phenyl-phenylacetyl ester melted at 96°C.

The acidified aqueous solution of water soluble substances was neutralized. By the Van Slyke method, 0.23 mgm. of amino nitrogen, the molecular equivalent to the amount of sulfur was found. The remainder of the nitrogen was precipitated as a reineckate. The Molisch and ninhydrin test was negative.

One half of the aqueous solution was evaporated to dryness in vacuo. The residue was extracted repeatedly with absolute alcohol. After evaporation of the alcohol, glyceryl tribenzoate was prepared from benzoyl chloride. After recrystallization from methyl alcohol, the m.p. was 72° C. (uncor.). It was not changed by mixture with a known sample of glyceryl tribenzoate. The analysis for carbon and hydrogen checked with that for pure substance.

Samples of provisual red from four batches were hydrolyzed by mild saponification. The products were determined and are given in table 1. The ratio of the petroleum ether soluble acid to ether soluble fatty acid is 5:1 and the ratio of visual red to fatty acid is about 2:1. Samples 1 and 2 were fresh and 3 and 4 were old samples.

Preparation of visual red. Visual red was prepared directly from provisual red and also from the residue, marked A, as indicated in the preparation of provisual red. The residue was dissolved in the smallest possible quantity of al-

cohol and potassium hydroxide was added to make a 5 per cent solution of alkali. After two hours at 27° C. the solution was extracted with petroleum ether. The extract was cooled to -20° C. to precipitate the excess sterids. The remaining cholesterol was precipitated by adding an alcoholic solution of digitonin. The supernatant solution of petroleum ether and alcohol was evaporated and the orange colored residue weighing about one gram was dissolved in anhydrous ethyl ether leaving the digitonin as a residue. After evaporation of the solvent the lipid was dissolved in petroleum ether. The solution was passed over a chromatograph tube containing calcium hydroxide. The red and yellow layers were elutriated with a solution of 3 per cent alcohol in petroleum ether. The pigments were refractioned separately by chromatograph absorption. The yield was about 80 mgm. of visual red and about 60 mgm. of yellow pigment (visual yellow) for each batch of 10,000 eyes.

TABLE 1
Alkaline hydrolysis of provisual red

	SAMPLE							
	1		2		3		4	
	mgm.	ratio	mgm.	ratio	mgm.	ratio	mgm.	ratio
Provisual red.....	193		312		165		663	
Visual red.....	1.08	13.5	174	12.5	65	4.3	159	2.2
Pet. ether sol.....	39	4.9	70	5	69	4.6	354	5.0
Ether sol.....	8	1	14	1	15	1	71	1
Water sol. (diff.).....	38		54		16		79	

A band containing a trace of a second red lipid was obtained at the top of the first chromatograph tube. An attempt to purify the few micrograms was unsuccessful. No definite evidence of lycopene, carotenes, or xanthophyll was obtained.

Properties of visual red. Visual red was a viscous amorphous lipid. It was very soluble in chloroform, petroleum ether, acetone, ethyl ether and glacial acetic acid. It was fairly soluble in absolute alcohol, almost insoluble in 85 per cent aqueous ethyl alcohol and insoluble in water and in one per cent aqueous solution of digitonin and sodium glycocholate. One per cent aqueous solution of sodium desoxycholate dissolved a trace. With antimony trichloride in chloroform, visual red gave a bluish purple color with a maximum spectral absorption at 480 mμ. An alcoholic solution of visual red showed no fluorescence under ultra violet light.

Analysis of visual red. Micromolecular weight determinations using camphor as the solvent gave a value of 530, with cyclopentadecanone, 518.

$C_{37}H_{60}O_2$. Calc. C 82.83. H 11.19

$C_{36}H_{58}O_2$. Calc. C 82.76. H 11.11

Found: C 82.26, 82.63, 82.26—H 11.74, 11.24, 11.74.

The test for nitrogen, phosphorus and sulfur was negative. Microhydrogenation in absolute alcohol resulted in 1.86 and 1.87 per cent absorption of

hydrogen and a loss of spectral absorption above 300 m μ . A film of visual red weighing 12.9 mgm. absorbed 21.7 mgm. of bromine vapor. A solution of iodine in alcohol produced a red precipitate of iodide. Alkaline hypiodite gave no iodoform. Treatment with Girard T reagent indicated the presence of carbonyl groups. A semicarbazide, m.p. 168–170 C. uncorrected, was prepared but the quantity was insufficient for analysis. Chromic oxidation followed by distillation of the acetic acid indicated 7.68 and 11.09 per cent methyl to carbon grouping.

DISCUSSION. Provisual red corresponds to transient orange of Lythgoe which was probably a mixture of colored lipids. Provisual red behaves as a glyceryl ester which after alkaline hydrolysis gave rise to two unsaturated fatty acids in the ratio of 5:1, a non-acidic visual red, glycerol and unknown water soluble substances containing nitrogen and sulfur. The fatty acid in the highest ratio was probably oleic acid. It is doubtful that the substances containing nitrogen and sulfur are impurities.

Visual red, an uncrystallizable lipid, has the properties of a carotenoid derivative such as lipid nature, unsaturation, spectral absorption, antimony trichloride reaction, and formation of insoluble iodides. The absorption of hydrogen and bromine and the absorption spectra indicates 5 or more vinyl groupings. The chromic acid oxidation forming acetic acid if compared with that of carotenoids suggests about 3 methyl vinyl groups. The reaction with Girard T reagent and the formation of a semicarbazine shows the presence of a carbonyl group. The absorption spectra by the shift to the longer wave lengths with polar solvents is evidence for the belief that a carbonyl group is adjacent to conjugated vinyl groups.

SUMMARY

1. Colored lipids, named provisual and visual red were isolated from bovine visual purple.
2. Preliminary data for the analysis for structures of these substances are reported.

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EXPERIMENTS TO ASCERTAIN THE EFFECT OF MANGANESE ON THE SYNTHESIS OF ASCORBIC ACID IN THE GUINEA PIG¹

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The inability of primates and the guinea pig to synthesize ascorbic acid within their bodies as other species are able to do is attributed by Rudra (1-3) to insufficient manganese in the tissues of these species. In support of his hypothesis Rudra reported that injection of mannose alone did not enable guinea pigs to synthesize ascorbic acid, but with a simultaneous injection of mannose and manganese accumulation of ascorbic acid resulted in certain tissues, notably liver and jejunum. Similar results were obtained when the experiments were conducted *in vitro*.

If one assumes that such increases in ascorbic acid are confined to the two tissues mentioned, and estimates total accumulation on the basis of 0.05 mgm. per gram (Rudra reported as much as 0.06 and 0.09, respectively, for liver and jejunum), a much larger value is obtained than the 0.165 mgm. critical dosage of ascorbic acid as reported by Gould *et al.* (4). However, Rudra states "The author does not . . . exclude the possible synthesis of non-specific indophenol-reducing substances" (2).

The method for the bioassay of antiscorbutic substances which was developed by Gould and co-workers offers a specific and sensitive procedure for investigating this postulated rôle of manganese in synthesis of ascorbic acid in the animal body. This report is concerned chiefly with results obtained by this method when mannose alone and mannose plus manganese, respectively, were injected into scorbutic guinea pigs.

PROCEDURE. Guinea pigs used in the experiment were obtained from the stock colony which was fed a commercial chicken feed *ad libitum*, skimmilk and succulent material such as Kentucky bluegrass, carrot tops, etc., depending upon the season. When the young animals attained weights of 250 to 300 grams, they were fed the scorbutogenic diet used by Sherman and co-workers (5) until their weights reached a maximum or started definitely to decline. Whereas the assay of ascorbic acid by the procedure of Gould *et al.* (4) requires that animals be highly standardized as regards concentration of blood phosphatase at the beginning of the test period, such standardization was not deemed essential for the work herein reported. Original plans required maintenance of animals on the scorbutogenic ration until blood phosphatase was below 5 units per 100 ml. of serum before beginning the series of injections. Later, however, it became desirable to ascertain whether mildly scorbutic animals would be able to utilize man-

¹ The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director.

nose more effectively in synthesis of ascorbic acid than those which showed symptoms of acute scurvy. Consequently, in 26 instances the phosphatase concentration was above 5 units per 100 ml. of serum at the beginning of the test period. A number of animals were used in 2 or even 3 tests, a procedure in keeping with Gould's observation that guinea pigs may be used repeatedly for ascorbic acid assay. Between any two test periods, however, such animals were first fed stock ration until marked recovery was apparent and again rendered scorbutic for the succeeding test.

Blood for phosphatase assay was obtained by heart puncture while the animals were under light ether anesthesia. Three-fourths to 1 ml. was withdrawn per bleeding since this amount was required to furnish adequate serum for duplicate determinations of inorganic phosphorus before and after action of phosphatase. Phosphorus was determined by a micro-modification of the Fiske-Subbarow method (6) after preparation of the samples by a procedure closely resembling that of Gould and co-workers. The colors were read in a Klett-Summerson photoelectric colorimeter with filter 66.

Intraperitoneal injections of the various solutions were begun on the day following the initial bleeding and were continued for either 3 or 7 successive days. All solutions contained 0.95 per cent sodium chloride in water. In those containing mannose the concentration was 80 mgm. of the sugar per ml. Manganese, when incorporated into the solutions, was added as the chloride. This element was injected in 3 different concentrations, viz., 0.5, 1 and 5 mgm. of the element per milliliter. The volume of solution injected daily was either 0.5 or 1 ml., the amount being uniform throughout a given test period.

In addition to determining the effect of injected mannose, with and without manganese, upon the concentration of serum phosphatase, an attempt was made to check Rudra's work on the effect of such injections upon reducing substances in livers of guinea pigs. In one series the animals were depleted of ascorbic acid by feeding a scorbutogenic ration for several days before using them. In the second series well nourished stock animals were used. Five hours after receiving the intraperitoneal injections the animals were killed and their livers analyzed for ascorbic acid by the procedure of Bessey and King (7).

RESULTS. In order to compare animals which were relatively uniform as regards initial concentration of enzyme, a concentration of 5 units of phosphatase per 100 ml. of serum was arbitrarily chosen as the dividing line between low and high phosphatase values. Consequently, in table 1 a group of animals receiving any series of injections usually is further divided into subgroups L and H, low and high initial phosphatase, respectively.

In group 1, where the animals received 3 injections of 40 mgm. of mannose only, the concentration of phosphatase dropped approximately 60 per cent during the 4-day period. The one animal with an initial value of 6.79 behaved as did the other five whose initial concentrations averaged only $\frac{1}{3}$ as much. In group 2 the 11 animals in subgroups L and H were given the regular number of injections, viz. 3, whereas those in subgroup A received 7 consecutive daily injections containing 0.25 mgm. of manganese in addition to 40 mgm. of mannose. Two of

the low phosphatase animals in group 2 showed positive responses, averaging 35 per cent increase, whereas a 69 per cent decrease in the concentration of the enzyme was observed in the 7 remaining animals and a slightly greater decrease, 74 per cent, in the two animals of group 2-H. The animals of group 2-A with

TABLE 1

Effect of injections of mannose and mannose plus manganese upon serum phosphatase of scorbutic guinea pigs

GROUP NUMBER	INJECTIONS		TOTAL NUMBER OF ANIMALS	POSITIVE RESPONSE			NEGATIVE RESPONSE				
	Mannose	Mn		Number of animals	Average phosphatase			Number of animals	Average phosphatase		
					Initial	Final	In- crease		Initial	Final	De- crease
	mgm.	mgm.			units/ 100 ml.	units/ 100 ml.	per cent		units/ 100 ml.	units/ 100 ml.	per cent
1-L	40		5	0				5	2.49	0.94	60
-H	40		1	0				1	6.79	2.93	57
2-L	40	0.25	9	2	1.79	2.42	35	7	1.86	0.57	69
-H	40	0.25	2	0				2	6.68	1.74	74
-A*	40	0.25	5	0				5	9.53	1.91	80
3-L	80		11	1	2.51	2.65	6	10	3.15	1.73	45
-H	80		8	1	8.17	14.00	71	7	9.30	2.81	70
4-L	80	0.50	17	7	1.90	3.31	74	10	3.18	4.74	49
-H	80	0.50	8	1	7.19	12.10	68	7	8.57	4.24	55
5	80	1.00	10	2	1.28	1.34	5	8	2.52	3.98	58
6	80	5.00	2	0				2	11.30	2.96	74

* This group was injected on 7 successive days rather than 3 as were all other animals.

TABLE 2

Effect of injected mannose, with and without manganese, upon ascorbic acid content of guinea pig livers

DAYS FED SCORBUTOGENIC RATION	MATERIALS INJECTED		ASCORBIC ACID IN LIVER	
	Mannose	Manganese	Total	Concentration
	mgm.	mgm.	mgm.	mgm./gm.
13	40	0	0.64	0.046
12	80	0	0.87	0.049
13	40	0.25	0.65	0.042
12	40	0.50	0.59	0.043
12	80	1.00	0.98	0.049
0	40	0	7.87	0.61
0	80	0	7.74	0.53
0	40	0.25	6.61	0.56
0	80	0.50	8.70	0.58

very high phosphatase levels initially were quite vigorous animals when the series of 7 injections were begun. Nevertheless they were unable to utilize mannose in the presence of manganese as is indicated by the drop in average concentration of the enzyme from 9.53 to 1.91 units during the 8-day period. Of the 19 guinea

pigs in group 3, which were injected with 80 mgm. of mannose daily, there was one in each subgroup which showed a rise in phosphatase, all others a marked drop. The animal in group 3-H which showed an increase in phosphatase from 8.17 to 14.00 during the first period, dropped after another series of 3 injections to 4.32 and then to 2.42 units per 100 ml. after a third successive period. However, of the 25 injected with 0.5 mgm. of manganese in addition to the sugar, group 4, there were 8 which showed a marked rise in phosphatase. In fact, 7 of the animals in 4-L showed an average increase of 74 per cent as compared with 10 for which there was a 49 per cent decrease. Of those showing increased phosphatase, however, two were practically lifeless when they were bled the second time. Dehydration may have influenced concentration of the enzyme in these two animals, although adult males did not show a similar increase in phosphatase when water was withheld for two days. It should be pointed out that the positive responses in group 4 were confined to a relatively short period of time when compared to the duration of the investigation, 9 months. Five of the positive responses in this group occurred within a period of 11 days although the injections and bleedings of the entire group were scattered over a period of 54 days. Six of the first 9 guinea pigs bled in group 4 were found to have higher concentrations after the injections than initially whereas only 2 of the last 16 behaved in this manner. Inability to detect positive responses throughout the entire period suggests strongly that inadvertently ascorbic acid became accessible in some way. Further evidence that synthesis did not occur is the fact that animals in which positive responses were noted were unable to maintain high levels of phosphatase when subjected immediately thereafter to a second series of injections of the same solutions. An example from group 3-H was cited previously. When injections of 80 mgm. of mannose together with 1 mgm. of manganese were given, 2 of the 10 animals barely maintained the original levels of phosphatase and the others showed pronounced decreases. The highest level of manganese injection, 5 mgm. daily, resulted in fatalities to 6 of the 8 animals into which these injections were made. The two which survived suffered an average of 74 per cent decrease in phosphatase during the period. When one considers all animals used in the investigation, it will be found that in 14 of the 78 individual test periods there was an increase in blood phosphatase and with two exceptions these were in animals receiving manganese injections.

Positive control experiments were conducted on 18 animals by injecting them on 3 successive days with 10 mgm. of ascorbic acid dissolved in 0.5 ml. of 0.95 per cent salt solution. With few exceptions the animals receiving ascorbic acid were those in which phosphatase had dropped to a low level during a period of injection with one of the mannose or mannose plus manganese solutions. In these 18 animals the concentrations of phosphatase rose during the test periods from an average initial value of 1.78 to 6.90 units per 100 ml. of serum, an increase of 288 per cent. Regardless of previous physical condition all animals given ascorbic acid showed pronounced increases in phosphatase values.

Repetition of Rudra's experiments, in which livers of guinea pigs were analyzed for ascorbic acid 5 hours subsequent to intraperitoneal injection with mannose

or mannose plus manganese, failed to show appreciable differences due to manganese. In table 2 it is to be noted that in livers of 2 animals injected with mannose after being fed a scorbutogenic ration for 12 or 13 days, the concentration of the vitamin averaged 0.048 mgm. per gram as compared with 0.045 for the mates which were injected with manganese as well as mannose. Similar results were obtained when livers of normal guinea pigs were analyzed, the average for each group being 0.57 mgm. per gram 5 hours after the injections.

SUMMARY

The concentration of blood phosphatase in scorbutic guinea pigs usually decreased during a series of injections with mannose solution regardless of whether manganese was contained in the solution.

Livers of guinea pigs injected with mannose and manganese contained the same amount of indophenol-reducing substances as those of animals injected with mannose only.

From these experiments it does not appear probable that injected manganese enables guinea pigs to synthesize ascorbic acid from mannose.

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CHANGES IN PROTEIN CONTENT AND IN SOME PHYSICO-CHEMICAL PROPERTIES OF THE PROTEIN DURING MUSCULAR ATROPHIES OF VARIOUS TYPES

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In a previous paper (1944) we reported that during muscular atrophy changes in the physicochemical state of the protein myosin, the probable substrate of the contractile mechanism (Bailey, 1942; Verzár, 1943; Dainty et al., 1944), can be demonstrated and that these changes can be retarded by appropriate electrical treatment or massage. However, the methods used in this earlier investigation permitted only the detection of the general trend of these changes, mainly a diminution of the myosin solubility, but did not permit any exact quantitative or qualitative comparison between the changes as observed in various types of atrophies. Furthermore, the experimental data did not reveal directly how far the diminution of the extractable myosin after atrophy is due to a true solubility change or to a diminished myosin content. However, from the data in the literature, the conclusion was drawn that changes in the myosin content alone could not explain the large diminution in extractable myosin.

No specific methods are available for direct determination of the myosin content of muscle. Myosin can be distinguished from other muscle proteins only by various physicochemical properties such as solubility in electrolyte solutions (Smith, 1934, 1937), viscosity (Dainty et al., 1944), birefringence (Muralt and Edsall, 1929, 1930), and others. With the advancement in physicochemical methods the definition of myosin has changed considerably (Dubuisson, 1945) and the fraction of intracellular muscle protein claimed to be myosin has increased from about 25 per cent by the old method of fractioning by heat coagulation (Fürth, 1895; Yanagi, 1931) to about 70 per cent by exhaustive extraction with 0.5 M LiCl solution (Smith, 1937). Therefore, no ratio between true myosin content and myosin extractable with a certain method can be established. We can compare only the amount of myosin extracted in a more or less pure form with the total amount of the non-collagenous protein. It is generally assumed that the latter corresponds more or less to the total intracellular protein.

PROCEDURE AND METHODS. All experiments were performed on white rabbits (3-5 kgm.). The gastrocnemius muscles used for analysis were either normal at the time the animals were killed, or had been submitted for various lengths of time (up to 5 weeks) to immobilization of the extended knee and foot by a plaster of Paris cast reinforced by wiregauze, tenotomy, or nerve section.

To keep the number of these very time-consuming experiments as small as possible and still accumulate enough data for a valid statistical analysis, various

combinations were used, such as: one leg normal and one leg denervated, one leg leg denervated and one leg tenotomy, one leg normal and one leg tenotomy, and so on.

In ether narcosis, just before the animals were sacrificed, both Achilles' tendons were freed and connected with isometric levers. Special care was taken to keep blood losses and interference with blood supply as low as possible, for preliminary experiments have shown that disturbed circulation can affect the myosin solubility. For the same reason, fixation of the knee joints was achieved only by external means which in no way interfered with the circulation of the limbs. Maximal isometric contractions produced by tetanic stimulation with a Harvard coil were recorded on a smoked drum. The results of these strength measurements are not included in this paper, since they varied much more than all other properties measured, and since the means indicate for atrophic muscles, including denervation atrophy, a much higher strength per gram muscle than in normal muscle. Often the total muscle strength after denervation, despite a weight loss of 40 per cent to 45 per cent, was distinctly higher than that of the control muscle. On the other hand, treated atrophied muscles and trained normal muscles had, as one would expect, on the average a higher tension development per gram muscle than the untreated controls. Since we cannot exclude the possibility that the relatively high values for atrophied muscles might be caused by insufficient fixation, we feel a careful reinvestigation with better fixation of the limbs should be made before these data are published in detail.

After the strength measurements, the animals were killed and the gastrocnemii dissected. Since reproducible results in myosin extraction under various biological conditions can be achieved only if one adheres rigidly to one technique (Smith, 1934, 1937), our extraction procedure is described in detail. All other steps in the analysis of the muscles can be gathered from the data in table 1, which gives the results step by step for a rabbit with normal legs, except that the Achilles tendon was freed and five test tetani of half a second duration applied to one side, while the other side was not touched at all. This type of control experiments was necessary, since in preliminary experiments, 5 minute continuous tetanisation produced a rather distinct myosin solubility diminution in confirmation of the work of Deuticke (1930, 1932), Kamp (1941), and Dubuisson and Jacob (1945). The results of all experiments of the type, as presented in table 1, demonstrated that the few short test stimuli have no appreciable effect upon the myosin solubility. The lines in *italics* in table 1 indicate which of the many data gained in each single experiment have been used for the summarizing in table 2.

The mincing of the muscles was performed on ice-cooled glass plates with sharp curved scissors as quickly as possible. Both muscles were mashed simultaneously and as evenly as possible. This gave more uniform results than any mechanical means of mashing we tried out. As soon as the muscle mash had the appearance of a thick paste, the main portion of each mash was extracted with 5 times its volume of 0.5 M LiCl and 0.03 M NaHCO₃ for exactly one hour under constant agitation by a stirrer and cooling by a mantle filled with ice and water. It is of utmost importance that agitation and temperature are kept as constant as pos-

sible in all experiments. Probably due to the fact that we extracted at a low temperature, our extracts never shifted to the acid side during extraction, and it was unnecessary to add NaHCO_3 constantly, as proposed by Bailey (1942). Immediately after extraction, the extract was separated from the extracted muscle mash by centrifuging and filtering through three layers of cheese cloth. Measured amounts of the extract were diluted slowly at a constant rate by release from a burette into 20 times its volume of a 0.00067 M phosphate buffer solution

TABLE 1
Rabbit, male, 2 560 grams

	LEFT GASTRO- NEMIUS (TENDON NOT FREED, NOT STIMULATED)	RIGHT GASTRO- NEMIUS (TENDON FREED, STIMULATED AS USUAL)
Muscle weight in grams.....	16.830	17.055
Muscle weight in % of animal weight.....	0.659	0.669
Mash used for Kjeldahl in grams.....	0.370	0.370
Mash used for collagen and dry weight determination in grams.....	1.730	1.480
Mash used for extraction in grams.....	14.730	15.205
Total N in mgm. per gram muscle.....	33.27	33.50
Total N in mgm. per cc. extract.....	2.88	2.86
NPN in mgm. per cc. extract.....	0.571	0.582
NPN in mgm. per gram muscle.....	3.4	3.50
Total protein N in mgm. per cc. extract.....	2.31	2.28
Per cent protein in extract.....	1.400	1.382
Extractable protein in % of muscle.....	8.40	8.29
Total protein in % of muscle.....	18.00	18.15
Collagen in % of muscle.....	3.96	3.86
Non-collagenous protein in % of muscle.....	14.04	14.29
Dry weight in % of wet weight of muscle.....	25.33	24.94
Precipitated myosin in % of muscle.....	5.04	5.12
Protein in supernatant fluid in % of muscle.....	3.43	3.29
Collagen in % of total protein.....	22.0	21.3
Extractable protein in % of non-collagenous protein.....	60.1	58.8
Myosin in % of non-collagenous protein.....	35.8	35.8
Myosin in % of extractable protein.....	59.9	61.2
Weight of extracted mash in grams.....	20.780	21.635
H ₂ O absorbed in % of original mash.....	40.9	41.8
Cc. needed of 0.067 M KH_2PO_4 per 10 cc. extract for main- tenance of pH 6.9 at dilution.....	1.16	1.16

of pH 6.9. The pH was constantly measured during dilution with a glass electrode and maintained at pH 6.9 by titration with a 0.067 M KH_2PO_4 solution. After 24 hours in the ice box, the precipitated myosin was separated from the supernatant fluid by centrifuging and decanting, its volume measured, then redissolved by adding an equal volume of 1.0 M NaCl. The N content of the myosin solution and of the supernatant fluid was determined by a semimicro Kjeldahl method, and their respective protein content calculated from the ni-

trogen values according to Bailey's analyses (1937) of muscle proteins. Finally, the isolated myosin and the protein retained in the supernatant fluid were calculated as per cent of muscle assuming with Howe (1924) that the same concentration of extractable protein existed in extract and in the extracted muscle.

All N determinations mentioned in this paper were done by the same semimicro method, and the collagen was determined according to the method of Spencer et al. (1934). Besides the data given in table 1, in each experiment, the protein content of the extracted muscles was determined, and from it and from the water content of the extracted muscle the protein still present calculated in per cent of the original muscle. For the final evaluation, only those experiments were used in which the sum of the protein in the extracted muscle and in the extract differed not more than ± 5 per cent from the protein amount found by direct analysis of the muscle mash.

RESULTS. In table 2 presenting the main results of our investigation, the data for each muscle type were treated as data gained by independent series. Only if, by such statistical treatment, the probability of a true difference between the corresponding data for two muscle types is doubtful, will there be a reference to a statistical analysis of the mean of the difference as found in those experiments in which the two conditions under consideration have been produced in the same animal. Such a careful statistical treatment is necessary because the various properties measured have, even in normal muscles, a very wide variation. In order to demonstrate clearly this extensive overlapping of the respective distribution curves, table 2 contains not only the means and their standard errors, but also the standard deviations from the means (in italics).

The distribution of the durations of the atrophies is approximately the same in all groups, thus permitting a valid comparison of the data for the various means listed. Generally speaking, the changes during denervation-atrophy and during atrophy due to tenotomy are in most respects the same, but somewhat more pronounced in the denervation atrophy. However, in immobilization-atrophy, fewer changes occur, and even these are less distinct than in both the other types of atrophy. The *weight loss* of 48 per cent following denervation is practically identical with the 46 per cent after tenotomy, while during immobilization the weight loss of 39 per cent is significantly less than the two other types of atrophy. *Dry weight* is not altered by atrophy due to casts, but is distinctly decreased, with statistical significance, for both the other atrophies. *Total protein* diminished 4 per cent by immobilization but 14 per cent by tenotomy or denervation. Since total protein decreases and *collagen* expressed in per cent of total protein increases 35, 49 and 66 per cent, the *non-collagenous protein* is reduced much more than is the total protein, namely, 12, 32 and 34 per cent for immobilization, tenotomy, and denervation respectively. The small difference for the last two conditions has statistical significance with 97/100 probability according to the data from the experiments in which the two conditions had been produced in the same animals. The amount of *myosin* isolated per gram muscle is decreased only 10 per cent by cast, but 68 and 76 per cent by denervation and tenotomy. For the two latter conditions the decrease in myosin is much larger than the decrease in

TABLE 2*

TYPE OF MUSCLE (22-30 DAYS)	NO.	MUSCLE WET WEIGHT IN % OF ANIMAL WEIGHT	DRY WEIGHT IN % OF WET WEIGHT	TOTAL PROTEIN IN % OF WET WEIGHT	COLLAGEN IN % OF TOTAL PROTEIN	NON-COLLAGENOUS PROTEIN IN % OF WET WEIGHT	MYOSIN IN % OF WET WEIGHT	EXTRACTABLE PROTEIN IN % OF NON-COLLAGENOUS PROTEIN	MYOSIN IN % OF NON-COLLAGENOUS PROTEIN	MYOSIN IN % OF EXTRACTABLE PROTEIN	HYDROLYTIC POWER IN % H ₂ O ABSORBED	NEUTRALIZATION CAPACITY IN C. KHPH ₄ NEEDED FOR pH 6.9	NPN IN MOL. WT. GR. WET MUSCLE
Normal muscle	34	0.624 ± 0.006 (±0.035)	24.78 ± 0.16 (±0.94)	18.08 ± 0.13 (±0.78)	20.5 ± 0.7 (±3.8)	14.88 ± 0.22 (±1.26)	4.75 ± 0.17 (±0.89)	80.4 ± 1.6 (±9.7)	33.9 ± 1.2 (±7.2)	55.2 ± 1.1 (±6.4)	44.6 ± 1.0 (±6.1)	1.33 ± 0.11 (±0.64)	3.24 ± 0.06 (±0.35)
Immobilization by cast	8	0.376 ± 0.032 (±0.090)	25.03 ± 0.36 (±1.00)	17.42 ± 0.19 (±0.55)	27.6 ± 0.6 (±1.8)	12.68 ± 0.24 (±0.69)	4.27 ± 0.33 (±0.83)	86.5 ± 3.2 (±9.1)	33.7 ± 2.9 (±8.4)	50.3 ± 2.1 (±6.1)	61.7 ± 3.9 (±11.0)	1.37 ± 0.26 (±0.72)	3.87 ± 0.09 (±0.24)
Tenotomy	12	0.338 ± 0.022 (±0.076)	23.40 ± 0.40 (±1.59)	15.56 ± 0.40 (±1.37)	30.5 ± 1.2 (±4.5)	10.79 ± 0.40 (±1.57)	1.53 ± 0.19 (±0.65)	39.4 ± 1.2 (±4.0)	13.9 ± 1.3 (±4.4)	35.3 ± 2.9 (±9.7)	38.8 ± 2.4 (±8.2)	2.28 ± 0.28 (±0.89)	2.98 ± 0.17 (±0.58)
Denervated	11	0.323 ± 0.019 (±0.067)	23.50 ± 0.31 (±0.99)	15.60 ± 0.24 (±0.80)	34.1 ± 1.5 (±5.0)	10.56 ± 0.34 (±1.18)	1.15 ± 0.06 (±0.21)	35.5 ± 1.8 (±5.9)	10.9 ± 0.7 (±2.4)	31.8 ± 2.7 (±8.8)	59.3 ± 5.8 (±19.1)	1.62 ± 0.16 (±0.62)	2.79 ± 0.13 (±0.44)

* Standard deviations in italics.

in non-collagenous protein due to the fact that not only a smaller fraction of the latter is extractable, but that also a smaller fraction of the extractable protein can be precipitated as myosin. By immobilization, however, the ratio extractable protein/non-collagenous protein is increased even as much as 10 per cent (statistically significant), while the ratio myosin/non-collagenous protein is unaltered, and the ratio myosin/extractable protein decreased by 9 per cent only (statistically significant).

The *hydrophylic power* of the muscle mash measured as per cent H_2O retained per gram original mash alters apparently quite independently of the protein changes. Although the latter are rather similar for tenotomy and denervation, the hydrophylic power decreases (13 per cent) by tenotomy but increases (33 per cent) by denervation. Immobilization with its relatively insignificant changes in protein increases hydrophylic power by 38 per cent. The somewhat larger increase by immobilization in comparison with denervation does not constitute a difference of statistical significance.

The *neutralization capacity* of the extracts as measured by the amount of KH_2PO_4 needed per 10 cc. extract to maintain a pH of 6.9 is not altered by immobilization; but is increased much more by tenotomy (72 per cent) than by denervation (22 per cent).

The *non-protein nitrogen* decreases 8 per cent by tenotomy but 15 per cent by denervation. However, immobilization increases NPN by about 10 per cent. All these differences were small, but occurred constantly in the dependent series, and therefore they are statistically significant with a probability above 99/100.

The time course of the various changes due to atrophy was investigated only for denervation atrophy. Data from all denervation experiments were arranged in 5 groups, according to duration, 4-6, 8-9, 11-13, 20-24, and 27-29 days, each group containing at least 4 animals with one leg normal and one leg denervated. Figure 1 represents graphically the means of the various data for each group expressed in per cent of the corresponding value for the normal muscles. The diminution in myosin is relatively slow for the first week, but then diminishes even more quickly than the weight values. The loss in extractable protein follows a course similar to that of myosin, but the rate is much less. Total protein diminution progresses rather slowly. The collagen concentration, however, increases rather rapidly and apparently at a constant rate. NPN is increased for the first days but then decreases steadily. Hydrophylic power is increased considerably soon after denervation and increases only slightly with further atrophy. The increase in neutralization capacity has a course similar to that of the hydrophylic power.

DISCUSSION. The outstanding result of the investigations reported in this paper is the distinct difference between the protein changes during immobilization atrophy and those occurring during atrophy due to tenotomy or denervation, despite the fact that the weight losses do not differ much in all three types of atrophy. The weight loss due to denervation reported in this paper corresponds well with the data for rabbit in the literature (f.i. Langley and Kato, 1915; Chen et al., 1924). That tenotomy produces almost the same weight loss as

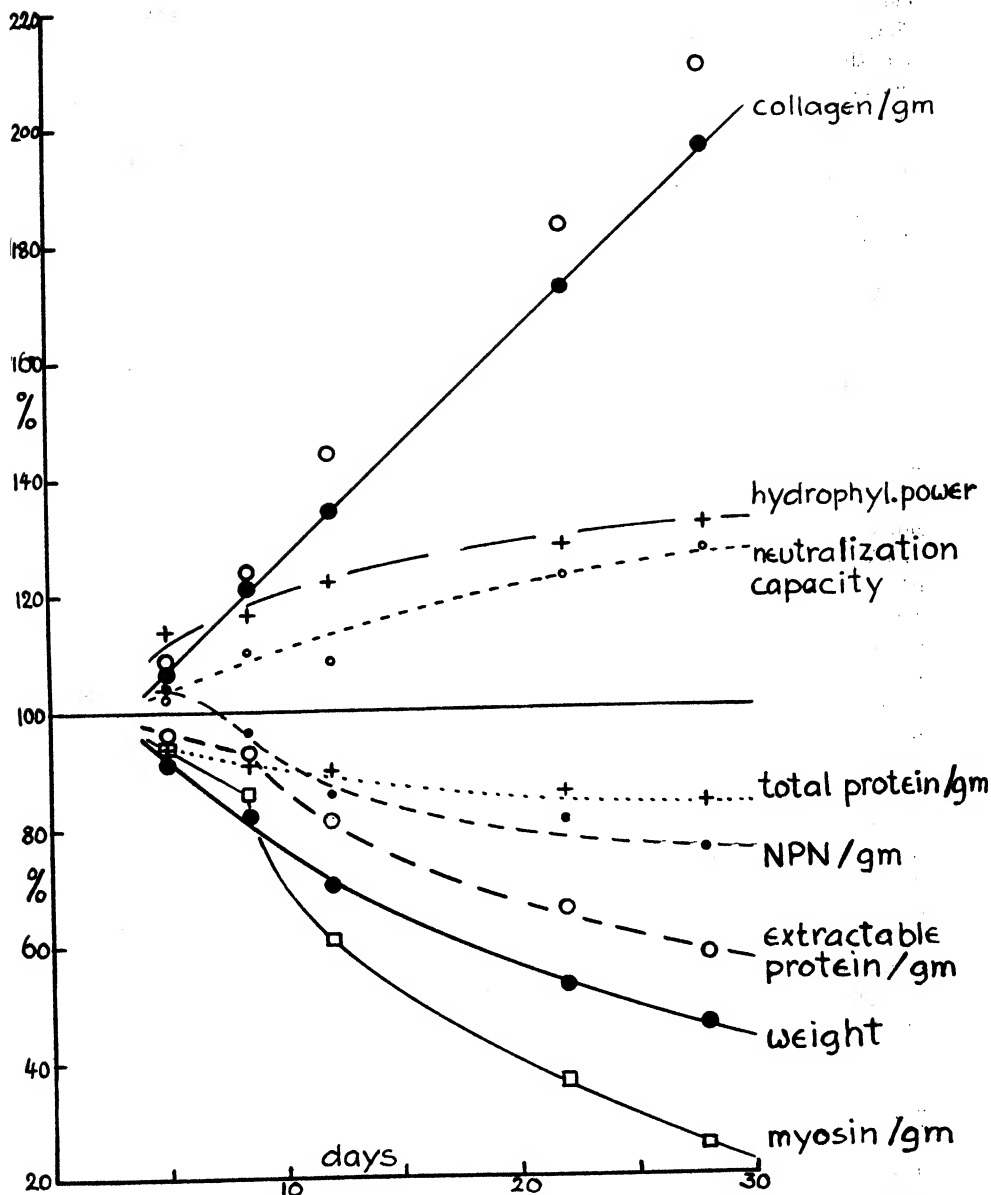


Fig. 1. Changes expressed in per cent of corresponding value for normal muscle in weight, total protein, collagen, extractable protein, myosin, hydrophilic power, neutralization capacity and in NPN with progressing denervation atrophy. Collagen concentrations calculated under the assumption that the total amount of collagen of a muscle does not alter during atrophy, are designated by circles above the dots for the experimentally found collagen concentrations.

denervation is in full agreement with the results of Lipschutz and Audova (1921). They found in rabbits the weight decrease after tenotomy only slightly smaller than that in denervation atrophy, so that a fair difference in weight between these two conditions becomes apparent only after three weeks and more. On the other hand, Lippman and Selig (1928) reported, also for rabbits, a 20 per cent higher loss in weight for tenotomy than for denervation. The same authors found very little weight loss after immobilization by cast. However, Usawa (1931), Thompson (1934), and Thomsen and Luco (1944) demonstrated in rabbits and cats the importance of the position of the fixated limb upon the course of the immobilization atrophy. The figures published by them for the extended position used by us correspond to our figures.

An approximately equal loss in relative dry weight of rabbit muscle after tenotomy and denervation as reported here has been observed by Audova (1923). We could not find in the literature comparable data for relative dry weight changes after immobilization by cast, but Grund (1913) found in dogs for disuse atrophy after amputation a less distinct increase in water content per gram dry weight than he had observed previously after denervation (Grund, 1912).

Grund also reports a larger decrease in protein content per wet weight of muscle following denervation than after amputation. In conjunction with our results, this suggests that disuse atrophy after amputation and disuse atrophy after cast are comparable to a certain extent. However, it must be pointed out that species differences might exist concerning the protein changes during atrophies. Avellone and Macco (1925) as well as Cahn (1927) reported distinct decrease in total protein after denervation of dog muscles, while under the same condition no protein changes were observed by Hines and Knowlton (1933) in rats and by Chor et al. (1937) in monkeys.

The collagen content in all three types of atrophy increases roughly reciprocally with the weight loss. This increase is rather similar to the collagen increase found by Spencer et al. (1937) in dystrophic rabbit muscles. As shown in figure 1, this increase in collagen/gram muscle progresses with uniform rate at least in denervation atrophy. This increase in collagen content is, however, smaller than the theoretical increase in collagen concentration calculated under the assumption that the total amount of collagen of a muscle does not change during atrophy (circles in fig. 1 above the dots for the experimentally found collagen concentrations). That such a deficit in total collagen of the whole muscle exists also during immobilization atrophy and after tenotomy can be calculated from the data given in table 2. This collagen deficit is of interest in regard to the question not yet decided by the histologists (see Tower, 1939) whether the observed proliferation of the connective tissue in atrophic muscle is only a relative or an absolute increase. The data presented here exclude the latter possibility and even indicate a certain absolute loss in connective tissue during atrophy.

The study of the time course in the various protein fractions during denervation atrophy revealed a break in the curves for extractable protein and for myosin. It is obvious from figure 1, that these two curves could have been drawn without

such a break. However, this other course of the two curves would indicate clearly that these two conditions start to change considerably later than all the other conditions investigated. Which of the two interpretations one prefers is not of much importance since both are in agreement to a certain extent with the previously reported findings (Fischer, 1940); namely, that in denervated rat muscles, birefringence decreases not at all or only very slightly during the first week or ten days, but decreases after this initial period rather progressively.

The first outstanding difference between atrophy due to cast and to the two other types of atrophy is the behavior of the ratio extractable/non-collagenous protein. This ratio increases somewhat in the former, but is strongly decreased in the two others. Hines and Knowlton (1933) demonstrated a small decrease in protein solubility for denervated rat muscle, by extraction with KCl. Westenbrink and Krabbe (1936) also reported a small decrease of KCl extractable protein in cat muscle after 18 days' denervation.

The second important feature in the protein changes after cast is the rather small decrease in the ratio myosin/extractable protein. The same ratio decreases four times as much after tenotomy and even more after denervation. A decrease of 30 per cent in this ratio for KCl extracts of cat muscle after denervation has been reported by Westenbrink and Krabbe (1936). But when comparing their figures with ours, one must keep in mind that they denervated only for 18 days prior to removing the muscles, and that their procedure gave a smaller ratio already for normal muscles. Steyrer (1903) found a distinct increase in the myosin/myogen ratio in press juice of denervated rabbit muscles by the heat fractioning method of v. Fürth. As pointed out before, the myosin determined with the old method is not identical with myosin as determined today; furthermore, experiments with press juice and with extracts are not quite comparable.

Our data reveal that only a small fraction of the original myosin can be isolated after denervation. However, we have at the moment no possibility to decide whether this is only due to a diminished solubility and to other physicochemical changes which diminish the amount of myosin that can be precipitated, or to changes in the chemical composition of the muscle protein beyond the relative increase in collagen. Cahn (1927) concluded from a rather small number of determinations of the arginine content of the non-collagenous fraction that changes in its chemical composition are produced by denervation. Recently Jacob (1945) demonstrated by electrophoresis that myosin from normal rabbit muscle is a mixture of 9-11 different proteins. Dubuisson and Jacob (1945) found only 6 electrochemically different proteins in frog myosin solution and they demonstrated that the diminished myosin solubility during fatigue is due to a formation of labile complex proteins by muscular activity. Szent-Györgyi and co-workers (see Dubuisson, 1945) were able to isolate, besides the myosin of Edsall, another soluble protein, the actin, which can combine with myosin to acto-myosin. The possibility that combination to complex proteins is partly the cause of the myosin changes reported in this paper cannot be excluded at the present.

The myosins precipitated in our experiments from normal and denervated muscles were more or less identical as measured by various physicochemical

properties. Gels of equal concentrations of both myosins have approximately the same refractive index; both show the phenomenon of double refraction of flow; and both could be spun into double refracting myosin threads. There was, however, one difference which came out clearly. By repeated precipitation by diluting and redissolving of myosin of normal muscle, we found, confirming Smith (1934), that a constant fraction of about 12 per cent will stay in solution each time. With myosin of denervated muscles this constant percentage was much higher. This observation has probably some relation to our earlier observation (1944) that a maintenance of an optimal pH range facilitates precipitation of normal myosin much more than precipitation of myosin from abnormal muscles.

The changes in hydrophylic power of the muscle mash are apparently a factor not depending alone on the protein solubility; even so we were able to confirm Ørskov's observation (1932) that fatigue increases swelling of muscle mash and simultaneously decreases protein solubility (Kamp, 1941). However, as can be seen from table 2, tenotomy decreases swelling despite a large decrease in protein solubility. A possible explanation for the decrease in hydrophylic power in tenotomy might be derived from the fact that in this type of atrophy the muscle fibers attain a much shorter length than in the two other types of atrophy. In consequence, the protein molecule chains are less straight, more folded or curled, and might even become entangled if this condition were maintained for some time. In such a configuration, less water might be absorbed due to a diminished free surface of the chain molecules.

The neutralization capacity of the extracts as measured in our experiments depends on many factors and it is impossible at the moment to estimate to what extent protein changes are responsible for any observed change in neutralization power. Smith (1938) found that only one-third of the buffering capacity of skeletal muscle in rigor is due to protein. Dubuisson (1941) reported an even smaller buffer capacity of the proteins at normal pH range. That protein alterations may play no rôle at all in the observed changes in neutralization capacity might be indicated by the statement of Dubuisson and Hamoir (1943) that there exists no significant difference between the electrotitration curves for normal myosin and myosin denaturated by heat or alcohol.

The decrease in NPN in denervation atrophy is partly due to the well known decrease in creatine (f.i. Cathcart et al., 1919; Avellone and Macco, 1925; Hines et al., 1942) and in phosphocreatine (f.i. Zanghi, 1928; Hines and Knowlton, 1933; Westenbrink and Krabbe, 1936; Levine et al., 1942). The initial increase observed during the first days of denervation is not statistically significant. However, the report of Palladin and Sigalowa (1934) that in the first days of denervation atrophy phosphocreatine is distinctly increased, indicates too that early changes in NPN might be in a direction opposite to the later changes. As long as no analytical data are available for changes in creatine and phosphocreatine changes after tenotomy, it might be permissible to assume a decrease in these two substances as the main factor for the decrease in NPN. No data could be found in the literature permitting any probable guess as to which sub-

stances might be responsible for the increased NPN in disuse atrophy due to cast in rabbits. Grund (1913) reported a decrease in NPN for disuse atrophy after amputation in dogs. The contradiction between these two observations might be due to a difference between these two types of disuse atrophy and/or to a species difference.

SUMMARY

The total protein concentration in gastrocnemius muscles of rabbits diminishes by about 14 per cent during denervation atrophy of 22 to 30 days' duration. In atrophy due to tenotomy of the same duration, the weight loss and the loss in total protein concentration are practically the same as in denervation atrophy. In atrophy due to cast in extended limb position, the weight loss is nearly as large as in the two other atrophies, but the loss in total protein concentration is only about $\frac{1}{3}$ of the loss in the other atrophies. Collagen concentration increases in all three types of atrophy about reciprocally with the weight loss. However, the increase in collagen content is somewhat less than expected under the assumption that the amount of collagen present in the whole muscle remains constant. In consequence of the increase in collagen content, non-collagenous protein content is decreased by 13, 25 and 29 per cent for immobilization, tenotomy, and denervation respectively. The amount of precipitable myosin per gram muscle is diminished for immobilization only by 11 per cent, but decreased by 69 and 78 per cent for tenotomy and denervation. This large decrease in myosin for tenotomy and denervation which is much larger than the decrease in non-collagenous protein, is due to the fact that in comparison with normal muscle a much smaller proportion of the protein can be extracted from the muscle, and a smaller fraction of the extractable protein can be precipitated as myosin. The hydrophylic power of the muscle mash is increased by about 35 per cent for immobilization and denervation, but decreased by about 13 per cent for tenotomy.

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THE EFFECT OF DAILY ELECTRICAL STIMULATION OF NORMAL AND DENERVATED MUSCLES UPON THEIR PROTEIN CONTENT AND UPON SOME OF THE PHYSICOCHEMICAL PROPERTIES OF THE PROTEIN

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In a preceding paper Fischer and Ramsey (1946) reported that distinct changes in protein content, in protein composition and in certain physicochemical properties of the protein occur during muscular atrophy due to denervation. In a simultaneous series of experiments, denervated as well as normal rabbit muscles had been submitted to daily electrical treatment or training for a period of 22 to 30 days. The data concerning protein content, composition and properties found for these muscles are reported here and compared with the data for non-treated muscles.

PROCEDURE AND METHODS. All general experimental procedures and methods were the same as reported in the preceding paper. In most of the experiments a Harvard coil was used as stimulator. In a few experiments a mechanical square wave stimulator, which permitted separate adjustment of strength, frequency, and duration of a single square wave, was employed. The results were practically the same for both types of stimulation. Training or treatment was given twice daily, consisting of 40 to 60 tetani of 1 to 2 seconds, duration, with appropriate rest periods. The stimuli were adjusted during each session to remain just maximal. During training or treatment, the animals were fastened to an animal board with the legs fixed in such a way that stimulation resulted in either an isometric tetanus in a position of about maximal length of the gastrocnemius, or in a free shortening. These two conditions are called in this paper "long" and "short" position.

Various combinations were used, such as: both legs denervated but only one treated, both legs denervated but one treated in long and the other in short position, and so on. The statistical evaluation of the data presented here is the same as used in the preceding paper.

RESULTS. Table 1 summarizes the results for treated normal and denervated muscles, and includes for comparison the data for untreated muscles.

The changes brought about by denervation are all diminished with the exception of the decrease in dry weight, which is not affected at all. The diminution in the denervation changes is much less distinct for treatment in the short position than in the long position. The differences between the data for the long position and for the untreated denervated muscle are all statistically significant. The differences between the following data for short and long position are not statistically significant, even if calculated according to dependent series: the ratio extractable protein/non-collagenous protein, the ratio

TABLE 1*

TYPE OF MUSCLE (22-30 DAYS)	NO.	MUSCLE WET WEIGHT IN % OF ANIMAL WEIGHT	DRY WEIGHT IN % OF WET WEIGHT	TOTAL PROTEIN IN % OF WET WEIGHT	COLLAGEN IN % OF TOTAL PROTEIN	NON-COLLAGENOUS PROTEIN IN % OF WET WEIGHT	MYOSIN IN % OF WET WEIGHT	EXTRACTABLE IN % OF NON-COLLAGENOUS PROTEIN	MYOSIN IN % OF NON-COLLAGENOUS PROTEIN	MYOSIN IN % OF EXTRACTABLE PROTEIN	HYDROLYZABLE POWER IN % H ₂ O ABSORBED	NEUTRALIZATION CAPACITY IN CU. KHPd ₄ NEEDED FOR pH 6.9	NPN IN MG. PER GRAM WET MUSCLE
Normal muscle stimulated in short position	160	0.079 ± 0.009 (±0.028)	24.68 ± 0.18 (±0.58)	17.81 ± 0.19 (±0.60)	20.4 ± 0.6 (±1.9)	14.28 ± 0.32 (±1.01)	4.65 ± 0.42 (±1.34)	57.2 ± 4.6 (±14.4)	31.6 ± 2.5 (±7.9)	57.2 ± 1.9 (±5.9)	44.8 ± 1.8 (±5.7)	1.09 ± 0.09 (±0.29)	3.28 ± 0.13 (±0.40)
Normal muscle stimulated in long position	70	0.031 ± 0.010 (±0.028)	24.56 ± 0.23 (±0.60)	17.64 ± 0.33 (±0.88)	20.5 ± 0.7 (±1.9)	14.06 ± 0.45 (±1.20)	4.75 ± 0.53 (±1.41)	61.1 ± 5.3 (±14.0)	32.0 ± 3.5 (±9.4)	56.6 ± 3.3 (±8.8)	44.0 ± 1.5 (±5.9)	1.15 ± 0.21 (±0.57)	3.26 ± 0.17 (±0.56)
Normal muscle	34	0.024 ± 0.006 (±0.038)	24.78 ± 0.16 (±0.94)	18.08 ± 0.13 (±0.78)	20.5 ± 0.7 (±2.8)	14.88 ± 0.22 (±1.28)	4.75 ± 0.17 (±0.99)	60.4 ± 1.6 (±9.7)	33.9 ± 1.2 (±7.2)	55.2 ± 1.1 (±6.4)	44.6 ± 1.0 (±6.1)	1.33 ± 0.11 (±0.64)	3.24 ± 0.06 (±0.55)
Denervated	110	0.323 ± 0.019 (±0.067)	23.50 ± 0.31 (±0.99)	15.60 ± 0.24 (±0.80)	34.1 ± 1.5 (±6.0)	10.56 ± 0.34 (±1.19)	1.15 ± 0.06 (±0.21)	35.5 ± 1.8 (±8.9)	10.9 ± 0.7 (±2.4)	31.8 ± 2.7 (±8.8)	59.3 ± 5.8 (±19.1)	1.62 ± 0.16 (±0.69)	2.72 ± 0.13 (±0.44)
Denervated and stimulated long position	100	0.441 ± 0.014 (±0.045)	23.59 ± 0.27 (±0.85)	16.70 ± 0.30 (±0.94)	26.8 ± 1.7 (±4.8)	12.21 ± 0.35 (±1.11)	2.06 ± 0.21 (±0.68)	41.6 ± 1.5 (±4.7)	16.8 ± 1.5 (±4.7)	39.8 ± 3.0 (±9.4)	47.5 ± 3.4 (±11.1)	1.50 ± 0.14 (±0.44)	3.06 ± 0.10 (±0.80)
Denervated and stimulated short position	100	0.392 ± 0.014 (±0.043)	23.21 ± 0.21 (±0.67)	15.88 ± 0.24 (±0.75)	28.4 ± 2.0 (±6.8)	11.38 ± 0.40 (±1.26)	1.67 ± 0.16 (±0.60)	39.3 ± 1.3 (±5.9)	14.3 ± 1.4 (±4.5)	37.4 ± 2.8 (±9.0)	47.8 ± 2.4 (±7.6)	1.56 ± 0.16 (±0.58)	2.84 ± 0.11 (±0.54)

* Standard deviations in italics.

myosin/extractable protein and the hydrophylic power. However, all these differences are in the same direction indicating also that the long position is more effective in diminishing the changes brought about by denervation.

In contrast to the higher effectiveness of the long position in electrical treatment of denervated muscle, no training effect could be observed for normal muscle in long position. In short position, a training effect was present only upon the weight of the muscles. The decrease observed for both positions in dry weight, total protein, and non-collagenous protein is statistically not significant, and if real, would indicate only a very slight edema of the muscles.

DISCUSSION. The effectiveness of electrical treatment upon the weight loss in denervation atrophy has been well established in recent years (f.i. Grodins et al., 1942, 1944; Wehrmacher et al., 1945; Hines et al., 1945). The data reported here demonstrate clearly that not the weight loss alone is retarded, but also that the deterioration of the myosin is appreciably delayed. The diminished decrease in relative dry weight and in NPN as found by us in the treated muscles confirms the observations of others concerning water content, creatine and phosphocreatine concentration (f.i. Martino, 1931; Hines et al., 1943; Wehrmacher et al., 1945). The higher effectiveness of the "long" position for the electric treatment of denervated rabbit muscles corresponds to the observations of Hines et al. (1944) that electrical treatment of denervated rat muscle is effective provided the muscles are weighted and stretched during the induced contractions. The same condition has been reported by Eccles (1944) as the most effective one for the electrical treatment of disuse atrophies.

The ineffectiveness of electrical training of normal muscle in the long position was surprising at first. However, careful study of the literature concerning effective electrical muscle training revealed that the training conditions always corresponded to our short position (f.i. Siebert, 1929; Vannotti and Pfister, 1933; Guckelberger and Keiser, 1938). Even in our short position, the training effects upon the weight as observed by us was much smaller than those reported in the literature. This might be due to the rather short duration of the single training session. For this reason we might also have missed a possible increase in NPN due to the distinct increase in creatine (Embsden and Habs, 1927; Palladin and Ferdmann, 1928) and in phosphocreatine (Ferdmann et al., 1929; Palladin et al., 1931). For the same reason, our experimental results do not permit the conclusion that electrical training does not affect the protein composition of normal muscle. We have only demonstrated that one type of electrical stimulation which is effective in retarding protein deterioration in denervated muscle has no effect upon the protein composition of normal muscle.

SUMMARY

Daily electrical treatment of denervated rabbit muscles is effective in retarding weight loss and deterioration of muscle protein. Treatment applied to a leg in flexed position, so that the muscle contracts against high resistance during stimulation, is much more effective than treatment in an extended limb position, in which the muscle contracts freely against no or little resistance.

With the same type of electrical stimulation, a training effect upon normal muscles was produced only if the stimulation was applied in the extended position. However, even under this condition, the training effect was restricted to an increase in muscle weight, and no changes in the protein composition became apparent.

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SPONTANEOUS RECOVERY OF MUSCLE FOLLOWING PARTIAL DENERVATION¹

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In previous experiments (Weiss and Edds, 1945), we found indications that when the major portion of the motor nerve supply of a muscle is removed, stimulation of the remaining motor fibers eventually yields disproportionately strong contractions.² The explanation had to be sought either in a marked hypertrophy of the still innervated muscle fibers or in the taking over of part of the denervated fibers by sprouts from the residual intramuscular nerve branches. The present experiments were undertaken to decide this alternative and to obtain quantitative data on the spontaneous improvement of partially denervated muscle in the absence of regeneration of the severed nerve fibers.

The plurisegmental origin of most limb nerves makes section of one or more of the contributory segmental roots the most effective method for partial denervation. The method had been used previously (Hines, 1942; Weiss and Campbell, 1944), but met with complications from some unchecked nerve regeneration. With the added precautions described below, it proved fully satisfactory. The muscles innervated by the sciatic plexus of the rat receive their nerve fibers through the ventral roots of L4, L5, and L6 in varying proportions. Since, as a rule, muscle fibers possess only a single motor nerve ending (literature in Fort, 1940), each root is connected with a different group of muscle fibers. Section of all three roots produces complete denervation. Assuming a relatively constant ratio of root contributions to any given muscle, the effects of cutting one root in one animal, and the other two roots in a second animal, should add up to the effect of total denervation done in a single animal. If a muscle after partial denervation changes less than it ought to according to the rule, this would prove that the effects of partial and total denervation are not directly comparable. Our experiments followed this general plan. In pairs of animals, one was subjected to unilateral section of the spinal root L5, and the other of L4 and L6 (henceforth designated as L4, 6). The leg muscles were examined at various intervals after the operation and compared both with normal controls and muscles that had been completely denervated for the same period of time. The examinations included *a*, muscle weight; *b*, isometric tension after direct and

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²In a personal communication, Dr. H. M. Hines mentioned similar observations after partial denervation of muscle. His remark partly motivated the present study. He has in the meantime published a preliminary report (Wehrmacher and Hines, *Fed. Proc.*, **4**: 75, 1945).

indirect stimulation; *c*, histological study. While all major leg muscles were studied in some detail, the *m. soleus* was selected for more comprehensive analysis.

MATERIALS AND METHODS. All experiments were done with the sciatic plexus of white rats (150–300 grams). Of the spinal nerves contributing to the sciatic nerve, L5 is usually the strongest and rather constant in size. L4 and L6 are shared between the sciatic and the lumbar and pudendal plexuses, respectively, and their contributions are more variable. The animals were operated and biopsied in pairs. To offset the size variations in the lumbar nerves, pairs of animals were selected in which the relative contributions of the three roots to the sciatic plexus were similar. The plexus was approached through a paramedian incision from the third lumbar to the third sacral vertebrae, severing the attachments of the abdominal muscles to the lumbo-dorsal fascia and the ilium. By separating fibers of the iliacus and psoas major muscles and retracting the ilium dorsally, the entire left sacral plexus was exposed without removing any bone or muscle tissue. The chosen nerves were then identified, transected and capped with a solution of methylmethacrylate in acetone to prevent regeneration (Edds, 1945). In a few animals, the partially denervated plantar extensors were deprived of the antagonistic action of the dorsi-flexors by cutting the peroneal nerve at the knee.

The animals were biopsied from 8 to 120 days after the operation. Under nembutal anesthesia, the maximum isometric tensions of the gastrocnemius and soleus muscles (tibial innervation) and of the tibialis anticus and extensor digitorum longus muscles (peroneal innervation) of both the operated and contralateral control leg were determined kymographically. In order to preserve circulation, only the tendons and the origins of the muscles were exposed. Indirect stimulation was applied from the tibial and peroneal nerves above the knee. For direct stimulation, the muscle was pierced with two needle electrodes at its origin and insertion. After the recordings, the following muscles, experimental and controls, were dissected and weighed: *Mm. gastrocnemius lateralis*, *gastrocnemius medialis*, *plantaris*, *soleus*, *tibialis anticus* and *extensor digitorum longus*. The soleus muscles were then fixed under even tension in chrome-sublimate solution, sectioned transversally and stained in Mallory's phosphotungstic acid hematoxylin or azan connective tissue stain. The motor nerves to the soleus muscles were fixed in osmic acid vapor for cross sections to show the ratio of degenerated to intact nerve fibers. Finally, the operated sacral plexus was re-exposed as a check of both the completeness of the original operation and any possible abnormalities in the distribution of the plexus components.

Sizes of muscle fibers were compared microscopically in sections of experimental and control soleus muscles. A normal soleus muscle of a 200 gram rat contains cca. 2000 fibers. Since this muscle is of the fusiform type, with all fibers extending over its full length, a cross section through the middle shows all fibers at their maximum width. In most of the specimens, muscle fiber diameters were measured directly with an ocular micrometer. For greater accuracy, some

of the muscle sections were projected on drawing paper, and the fiber outlines were traced and measured with a planimeter. Up to one month postoperative, the size range of denervated atrophying fibers is still close to the lower range of normal fibers. In older cases, however, the denervated fibers can be clearly distinguished by their small sizes (figs. 3 and 4). Complete counts of intact and denervated fibers could thus be made.

The nerve fibers in the soleus nerves were counted directly under oil immersion.

EXPERIMENTAL. The animals recovered quickly from the operation and began to use the legs on the operated side within a few days. Co-ordination was not noticeably impaired, except for loss of strength. The biopsies proved that the operation had been successful in all cases; the lumbar nerves had been correctly identified and the methacrylate caps prevented any regeneration of the severed nerve fibers into the distal stumps (Edds, 1945).

Weight loss following complete denervation. In seven animals, the sciatic nerve of one side was cut and the six major leg muscles of both sides were weighed after periods of from 14 to 74 days. The graph, figure 1, gives the weights of these six muscles, expressed in per cent of their normal controls, plotted over the period of denervation. The graph shows that atrophy is nearly complete about 80 days after denervation, when the weight of the muscles has become stationary at cca. 22 per cent of the initial weight, which value represents the residual content of non-contractile tissue (blood vessels, fascia, connective tissue). According to Knowlton and Hines (1936), a denervated gastrocnemius muscle in the rat loses weight faster than is indicated by the above curve. The difference can be partly accounted for by the fact that the gastrocnemius contains a higher proportion of contractile tissue than the average of all six leg muscles on which our graph is based. It would be better for comparative purposes, to introduce a "half-time" value of degeneration, that is, the time at which a denervated muscle has lost just 50 per cent of its contractile tissue. In our cases, with a contractile fraction of $100 - 22 = 78$ per cent, half of this fraction (39 per cent) has disappeared when the muscle weight totals $39 + 22 = 61$ per cent, which occurs about the sixteenth day. This compares with a "half-time" of 13 days for the gastrocnemius as calculated from Knowlton and Hines' data.

Comparisons between totally and partially denervated muscles should be based on the weight losses of the contractile muscle fractions alone, after deducting the weight of the non-contractile portion. We have estimated the constant deduction for the soleus muscle as of the order of 25 per cent. The heavy aponeurosis along the posterior surface of the muscle accounts for this relatively high value. A sample soleus muscle after 74 days of complete denervation weighed 32 per cent of its normal control, and since atrophy after this period is nearly complete, the 25 per cent estimate for residual non-atrophying tissue is conservatively low.

The graph, figure 2, expressing weight loss of the contractile portion only of the soleus muscle, was computed on that basis. From the weight of each denervated muscle (W_d), an amount equalling 25 per cent of the weight of its control (W_c) was deducted, and the difference ($W_d - \frac{25}{100} W_c$), which is the actual weight

of residual contractile tissue in the experimental muscle, was expressed in per cent of the control. The smoothed curve gives the percentage reduction which the average contractile unit has undergone after a given period of complete denervation.

Weight loss after partial denervation. Sixteen animals with unilateral section of either L5 or L4, 6 were grouped in complementary pairs and sacrificed after periods of from 8 to 120 days. For a preliminary survey, the total weights of the six main leg muscles of the operated side were computed in per cent of the corresponding muscles of the control side of the same animals. They are listed in table 1. Also included are two pairs with additional transection of the peroneal nerve. The table reveals the following facts.

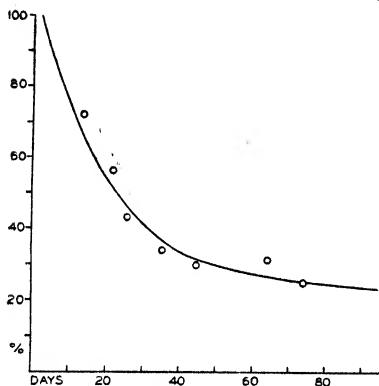


Fig. 1

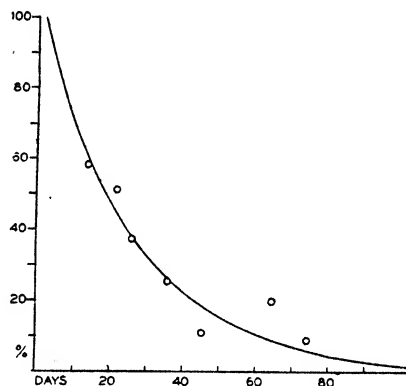


Fig. 2

Fig. 1. Progress of atrophy of six major leg muscles (gastrocn. lat., gastrocn. med., plantaris, soleus, tibialis ant., ext. digit. long.) after transection of the sciatic nerve.

Fig. 2. Weight loss of the contractile fraction only of denervated soleus muscles, calculated on the basis of 25 per cent non-contractile tissue content.

Elimination of L5 generally affects the plantar extensors (with the possible exception of the m. plantaris) more severely than it does the dorsi-flexors, while the reverse is true for L4, 6. However, the relative contributions of the various roots to any one muscle vary considerably, as pointed out for the leg muscles of the dog by Huddleston and White (1943).

If the denervated parts of these muscles had undergone the same progressive atrophy as after total denervation, their weights should have steadily declined with time. Instead, the figures of the table show not only no continued decline after the first week, but often a marked increase, returning the muscle to fully or nearly its original weight (= weight of the controls). Evidently, the presence of some residual innervation in the muscle has somehow arrested the atrophy of the denervated portion. We shall refer to this effect as the "compensatory reaction." A more detailed analysis of it follows.

We have found that the contributions of L5 and L4, 6 to the combined mass

of the leg muscles are of about equal size, with only a slight preponderance of L5. Transection of either L5 or L4, 6, therefore, denervates about one half of the contractile tissue, leaving the other half intact. If we designate the mass of the intact muscles as M_i , the mass of muscles completely denervated as M_d , and the mass of half denervated muscles as M_p , we can argue as follows. Let us

TABLE 1
Percentage weights of partially denervated muscles

CASE	DAYS P. OP.	RESID. INNERV.	MUSCLE WEIGHTS EXPRESSED AS PER CENT OF CONTROL					
			Gastroc- nemius lateralis	Gastroc- nemius medialis	Plantaris	Soleus	Tibialis anticus	Extensor digitorum longus
R748	8	4, 6	79	52	88	65	98	95
R744	8	5	68	79	67	70	76	104
R745	14	4, 6	73	58	93	82	92	100
R749	14	5	74	94	73	86	54	71
R765	22	4, 6	68	59	104	94	103	93
R764	21	5	75	89	72	90	49	73
R649	30	4, 6	47	103	62	97	88	55
R647	29	5	93	97	97	85	28	90
R654	44	4, 6	79	51	95	100	94	98
R651	44	5	86	110	103	92	39	103
R673	62	4, 6	95	44	96	77	87	88
R672	61	5	93	100	68	102	37	72
R669	103	4, 6	78	54	108	79	96	90
R666	102	5	98	104	100	111	65	105
R652	118	4, 6	95	53	113	89	98	91
R653	120	5	100	114	96	110	42	92
R772*	32	4, 6	76	24	93	100	(36)†	(46)
R771*	31	5	85	87	88	86	(32)	(44)
R741*	66	4, 6	92	62	97	66	(19)	(24)
R743*	65	5	75	83	75	71	(25)	(31)

* Cases with peroneal nerve transected.

† Figures in brackets refer to completely denervated muscles.

‡ Excessive value because of undersized control muscle; see table 4, column a.

first assume that the intact and denervated halves of these muscles behave independently, that is, each half as if it were one half of a fully intact or of a completely denervated muscle. Then, $M_p = \frac{M_i}{2} + \frac{M_d}{2}$. However, if contrary to our assumption, either the intact half ($\frac{M_i}{2}$) grows by hypertrophy, or the

denervated half ($\frac{M_d}{2}$) atrophies less than expected, or both, then $M_p > \frac{M_i + M_d}{2}$, with the magnitude of the inequality indicating the extent of the compensatory reaction. A comparison between observed M_p values and the calculated values of $\frac{M_i + M_d}{2}$ for various periods of denervation is given in table 2. Column *a* gives the total weight of the control muscles, M_i ; column *b*, the calculated weight of these muscles after total denervation, M_d , as determined from the

TABLE 2

Comparison of expected and observed weights of six leg muscles following partial denervation

CASE	DAYS	RESID. INNERV.	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>e</i>
			M_i	M_d	$\frac{M_i + M_d}{2}$	M_p	$100 \frac{d - c}{c}$
			grams	grams	grams	grams	per cent
R748	8	4, 6	2.077	1.660	1.869	1.588	-15
R744	8	5	1.509	1.206	1.358	1.196	-12
R745	14	4, 6	1.518	0.971	1.245	1.324	7
R749	14	5	1.805	1.155	1.480	1.363	-8
R765	22	4, 6	1.542	0.787	1.165	1.239	7
R764	21	5	1.676	0.888	1.282	1.230	-4
R649	30	4, 6	2.063	0.865	1.464	1.553	6
R647	29	5	1.461	0.629	1.045	1.144	10
R654	44	4, 6	1.577	0.489	1.033	1.245	20
R651	44	5	1.812	0.562	1.187	1.545	30
R673	62	4, 6	1.936	0.503	1.220	1.543	27
R672	61	5	2.308	0.624	1.466	1.811	24
R669	103	4, 6	2.204	0.484	1.344	1.769	32
R666	102	5	2.115	0.464	1.290	1.960	52
R652	118	4, 6	1.971	0.394	1.183	1.700	44
R653	120	5	1.921	0.384	1.153	1.742	51

graph, figure 1; column *c*, the values $\frac{M_i + M_d}{2}$; column *d*, the observed values of M_p ; and column *e*, the percentage excess of M_p over $\frac{M_i + M_d}{2}$.

It is evident from column *e* that after an initial slight deficit, there appears a marked excess, the magnitude of which tends to increase with time. These results prove that intact and denervated portions of a partially denervated muscle do not behave independently and cannot be treated in additive fashion. Either the intact portion actually increases in weight, or the denervated portion decreases less than after total denervation.

For closer study, the soleus muscle was singled out.

Nerve supply. The relative share of L5 and L4, 6 in the innervation of the soleus was determined from cross sections of the soleus nerve at its entrance into the muscle. The number of myelinated fibers present in the nerve at various intervals after elimination of either L5 or L4, 6 was compared with the number of

myelinated fibers on the intact control side. The normal nerve contains approximately 100 myelinated fibers. The average of ten animals studied from 1 to 6 weeks after operation shows that about 80 per cent of these fibers are derived from L5 and 20 per cent from L4, 6. The computations presented farther below are based on these figures.

During later stages of denervation an interesting variety of nerve regeneration intruded into the picture. Already on a previous occasion (Weiss and Campbell, 1944), it was noticed that the section of part of the sciatic roots did not entail a permanent deficit in the fiber content of the nerve. After some time, masses of regenerating fibers were seen in the nerve. It was taken for granted that these had come from the proximal roots, even though such an origin appeared highly dubious in view of the practice of evulsing the root stumps. In our present experiments, regeneration of the severed nerves was effectively suppressed by capping, and the absence of such regeneration was confirmed at autopsy and

TABLE 3
Numbers of myelinated fibers in the soleus nerve after section of L5

CASE	DAYS	NUMBER OF FIBERS*		
		Large (above 2μ)	Small (below 2μ)	Total
R748	8	19 (20)	2 (2)	21 (22)
R745	14	29 (28)	7 (7)	36 (35)
R765	22	9 (10)	3 (3)	12 (13)
R649	30	12 (12)	9 (9)	21 (21)
R654	44	6 (6)	13 (13)	19 (19)
R673	62	6 (5)	55 (45)	61 (50)
R669	103	6 (5)	57 (40)	63 (45)
R652	118	8 (7)	51 (46)	59 (53)

* Values in brackets give the ratio of fibers in per cent of the intact control nerve of the same animal.

histologically. Nevertheless, fibers, which can only be interpreted as regenerating ones, again appeared in the partially denervated nerve. They could be recognized by their smaller size and by the fact that several—up to four—were often contained in a single neurilemmal tube. The following table (table 3) lists the number of small ($<2\mu$) and large ($>2\mu$) fibers found in soleus nerves after elimination of L5.

As one can see from the table, there are only few small fibers present in these nerves at the beginning. Then, towards the end of the first month, their number starts to increase markedly, while the number of large fibers shows an equally significant drop. During the second month, the smaller fibers keep increasing in number up to between fifty and sixty, while the large fiber class remains stationary.

The trend of these figures is so consistent that the following interpretation seems inescapable. Evidently, some of the larger ones among the residual unsevered fibers of the sciatic nerve had suffered some belated damage, had

broken down within 20 to 30 days, and then regenerated again, undergoing some branching on the way. Therefore, it is a case of nerve regeneration, but from the surviving, not from the severed fibers. Pressure from the greatly swelling and degenerating fiber stumps, among which the intact fibers lie interspersed, may reasonably be assumed to be the cause of the damage. The soleus nerve of course, presents only a small sample of what has been occurring on a much larger scale in the whole sciatic. In the soleus nerve, about ten to fifteen large fibers succumbed, and in their stead some forty to fifty new small fibers appeared, indicating branching at an average of three times. The gradual increase in the count of small fibers between 22 and 62 days ($3 < 9 < 13 < 55$), in contrast to the more abrupt reduction of the larger fibers, indicates that the old fibers were not injured all at the same level, but at different sites, so that the regeneration distances varied and the regenerating branches arrived at the far distal counting level at different times. Since they do not reach the muscle in force until 2 months after the operation, they can be discounted as a source of the earlier compensatory changes.

Muscle weight. We know for each period of denervation a , the weight of the contractile fraction of the intact soleus muscles; b , the weight of the residual contractile tissue in completely denervated muscles; c , the ratio of intact to denervated tissue after the section of either L5 or L4, 6; d , the weight of the contractile fraction of the partially denervated muscles. These values were obtained as follows.

a is the weight of the control muscle of the animal in question, minus a deduction of 25 per cent for noncontractile tissue, according to the statements on page 589.

b is computed by reading the per cent of residual contractile tissue for the given period of denervation from the graph, figure 2, and multiplying by the weight of the control a .

c , according to the nerve fiber counts reported above, is 1:4 after section of L5, and 4:1 after section of L4, 6.

d is calculated by deducting from the gross weight of the partially denervated muscles, 25 per cent of the gross weight of their control muscles, as in a , for unchanged noncontractile substance.

Tables 4 and 5 list the results for the groups with severed L5 and L4, 6, respectively. Column c was computed on the basis of the 1:4 contribution ratio between L4, 6 and L5. Thus after section of L5 (table 4), there is left one-fifth innervated tissue, i.e., one-fifth of the weight indicated in column a , and four-fifths denervated tissue, which in totally denervated muscle, would amount to four-fifths of the figure in column b . The sums of these values, $\frac{a + 4b}{5}$, therefore, give the weights to be expected if denervated and innervated fractions behaved independently. These values are listed in column c . After section of L4, 6 (table 5), the corresponding c values are $\frac{4a + b}{5}$. Column e gives the excess in per cent of the actually observed weights d over those calculated in c .

It is clear from column *e* of table 4 that except for an initial deficit during the first week, the observed weights exceed the expected weights by progressively larger amounts. To judge from the rather stationary absolute weights (col-

TABLE 4
Soleus muscles after section of L5

CASE	DAYS	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>e</i>
		Intact muscle (contract. fraction)	Compl. denerv. muscle	$\frac{a + 4b}{5}$	Part. denerv. muscle	Compens. effect $100 \frac{d - c}{c}$
		grams	grams	grams	grams	per cent
R748	8	0.0840	0.0630	0.067	0.050	-25
R745	14	0.0795	0.0469	0.053	0.061	15
R765	22	0.0593	0.0255	0.032	0.054	69
R649	30	0.0660	0.0211	0.030	0.063	110
R654	44	0.0413†	0.0074	0.014†	0.041	193†
R673	62	0.0645	0.0052	0.017	0.045	165
R669	103	0.0653	0.0	0.013	0.042	223
R652	118	0.0720	0.0	0.014	0.061	336
R772*	32	0.0715	0.0207	0.0309	0.0715	132
R741*	66	0.1043	0.0063	0.026	0.057	119

* Cases with peroneal nerve transected.

† Control muscle of subnormal weight.

TABLE 5
Soleus muscles after section of L4, 6

CASE	DAYS	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>e</i>
		Intact muscle (contract. fraction)	Compl. denerv. muscle	$\frac{4a + b}{5}$	Part. denerv. muscle	Compens. effect $100 \frac{d - c}{c}$
		grams	grams	grams	grams	per cent
R744	8	0.0652	0.0489	0.062	0.039	-37
R749	14	0.0788	0.0465	0.072	0.064	-11
R764	21	0.0645	0.0290	0.057	0.056	-2
R647	29	0.0488	0.0161	0.042	0.039	-7
R651	44	0.0938	0.0169	0.078	0.084	8
R672	61	0.0930	0.0074	0.076	0.096	26
R666	102	0.0712	0.0	0.057	0.081	42
R653	120	0.0683	0.0	0.055	0.077	40
R771*	31	0.0795	0.0238	0.068	0.065	-4
R743*	65	0.1125	0.0067	0.091	0.070	-23

* Cases with peroneal nerve transected.

umn *d*) of the partially denervated muscles, either atrophy of the denervated fraction has been arrested at an early date, or hypertrophy of the intact fractions had compensated for the loss.

Table 5 shows the same trend, but less strikingly. The difference must be ascribed to the fact that in these muscles four fifths of the original mass had remained intact, as against only one fifth in the previous series; since the tables express both degeneration and compensation in relation to the total mass of the muscle, the relative effect is obviously the less, the more muscle tissue had been left innervated.

The negative values at 8 days p. op. are paradoxical, since they imply that these muscles have lost more weight than after complete denervation (compare column *d* and *b*). The case remains unexplained. The slightly negative values at later stages in table 5 are presumably due to the fact that the value of 80 per cent allocated to the contribution of L5 is slightly too high, and that a downward correction would have reduced the mass of residual intact tissue, hence the values in column *c*, accordingly. Considering the many estimates that had to enter our calculations, the results are, on the whole, of satisfactory consistency.

Since stretch has been shown to have a marked effect on the trophic state of muscle (Weiss, 1934; Eccles, 1944; Thomsen and Luco, 1944), it seemed desirable to examine to what extent the arrest of atrophy after partial denervation depends on functional activity. In four animals, elimination of L5 or L4, 6 was combined with transection of the peroneal nerve, paralyzing the dorsi-flexors. The plantar extensors were thus deprived of the periodical stretching normally produced by their antagonists. The results are summarized in the bottom lines of tables 4 and 5. The compensatory reaction was still pronounced in the animals lacking L5, while case R743 (L4, 6 removed) is definitely aberrant. The positive cases R772 and R741 prove that stretch is not an essential factor in the compensatory reaction.

Muscle strength after partial denervation. The maximum isometric tensions developed by control and experimental plantar and dorsi-flexor muscle groups, following stimulation of the muscles directly or through their nerves, were recorded in six pairs of animals with either L5 or L4, 6 cut, at intervals of from 8 to 120 days. The results of direct muscle stimulation, contracting both the innervated and denervated fibers, were in good agreement with the weight determinations. The tension of fifteen partially denervated muscles (gastrocnemius and soleus) averaged 78 per cent of that of their controls, which compares with an average weight ratio for the same muscles, after correction for the non-contractile fraction, of 79 per cent.

Since the compensatory reaction consists of an increase of the innervated muscle portion, by hypertrophy or reinnervation, indirect stimulation should produce increasingly stronger contractions as time goes on. This was verified as follows.

Having paired animals with matching plexus patterns, we can assume that the fractions of muscle left innervated after transection of L5 and L4, 6, respectively, are complementary, that is, add up to one total muscle set. Isometric tensions obtained by stimulating the remaining roots L5 in one, and L4, 6 in the other, should then add up approximately to the average of the tensions obtained from stimulating the whole intact sciatic nerves of the two control sides. A technical

point to be considered is that even in normal muscles the sum of tensions after separate stimulation of two roots is slightly larger than after simultaneous stimulation (for a review of the literature, see Fort, 1940), partly because of a small percentage of muscle fibers with two endings, and partly for mechanical reasons (Löwenbach and Markee, 1945). However, since the error from this source is evidently smaller than that introduced by matching different individuals, we may ignore it for the present purpose. The results of the measurements are listed in table 6. Column *a* gives the total isometric tensions of fully innervated leg muscles for control. The values represent the mean for each pair of animals. Column *b* gives the combined isometric tensions of the innervated fractions of the partially denervated muscles. These values were obtained by simple addition of the values recorded separately for each member of a given pair. Column *c* gives the excess of *b* over *a* in per cent.

The figures prove that except for the earliest period, the combined strength of the innervated fractions of pairs of complementary muscle sets greatly exceeds the strength of an intact single set. The effect is striking in the plantar extensors, but erratic in the dorsi-flexors. The reasons for this discrepancy are obscure. It is noteworthy that the tensions of complementary pairs have never added up to fully twice the control tension. This indicates that the experimental muscles have not recovered full strength within the period of observation. As will be described below, however, histological study showed even the oldest cases still in the process of improvement.

Histological observations. As outlined above, there are two possible explanations for the observed compensatory reactions. Either the muscle fibers with residual intact innervation have undergone compensatory hypertrophy, or some of the denervated fibers have received new nerve connections and thus regained their normal size and strength. The following histological observations have decided unequivocally in favor of the latter alternative.

Conclusions as to the response of muscle fibers to experimental changes are of questionable value, unless they are based on an adequate sampling of the whole fiber population. A statistical study of the variability of fiber size, the results of which will be reported on a later occasion, has demonstrated, for instance, that maximum fiber size alone is no reliable criterion of the trophic state of a muscle, since maximum size varies much more between individual fascicles than does mean fiber size; it is the latter that correlates with weight and strength. In order to illustrate how partial denervation affects the fiber population, we have selected some typical examples from our records. The graph, figure 3, shows representative sample fascicles from three different soleus muscles, with the fibers of each fascicle arranged in the order of size (diameter). The two fascicles in the left box are from a muscle 45 days after complete denervation; the four fascicles in the box at the right are from a muscle (R654), 44 days after partial denervation by section of L5; the three middle ones from the normal control muscle of this latter animal.

Comparing first the normal with the completely denervated muscle, it can be seen that their fibers fall into two sharply distinct classes, the former above, the

latter below, cca. 15 micra (dotted line). The ratio of the mean cross sections of the former over the latter (square of mean diameter ratios) is more the 5:1, which means that the denervated fibers have shrunk to an average of less than 20 per cent of their original weight, a value well in agreement with that extrapolated from the curve of weight loss of contractile tissue at 45 days (fig. 2). The fiber population of the partially denervated muscle is clearly intermediate

TABLE 6
Isometric tensions of complementary pairs of partially denervated muscle groups and their controls

CASE	DAYS	RESID. INNERV.	^a ISOMETRIC TENSIONS OF CONTROL MUSCLES AFTER INDIRECT STIMULATION: AVERAGES OF COMPLE- MENTARY PAIRS		^b ISOMETRIC TENSIONS OF PARTIALLY DENERVATED MUSCLES AFTER INDIRECT STIMULATION: SUMS OF COMPLEMENTARY PAIRS		^c EXCESS OF EXPERIMENTAL OVER CONTROL TENSIONS	
			Plantar extensors	Dorsi- flexors	Plantar extensors	Dorsi- flexors	Plantar extensors	Dorsi- flexors
			grams	grams	grams	grams	per cent	per cent
R748 R744	8 8	L4, 6 L5	1168		1000		-14	
R745 R749	14 14	L4, 6 L5	918	425	935	550	2	29
R765 R764	22 21	L4, 6 L5	1520	688	2200	710	45	3
R673 R672	62 61	L4, 6 L5	2150	515	2605	630	21	22
R669 R666	103 102	L4, 6 L5	1878	850	2893	855	54	0.6
R652 R653	118 120	L4, 6 L5	1540	888	2400	1030	56	16
R772* R771*	32 31	L4, 6 L5	1883		2470		31	
R741* R743*	66 65	L4, 6 L5	2125		4000		88	

* Cases with peroneal nerve transected.

between normal and fully denervated muscle. While some of its fascicles contain fibers of either kind only, the majority of fascicles are of the mixed composition exemplified in the graph. They contain variable proportions of *a*, fibers of normal size; *b*, fully atrophied fibers, and *c*, fibers of intermediate size. Their frequency distribution (humps of the enveloping contour curve) often shows them as belonging to three distinct classes, as can be seen in all but the second to the last fascicle of the graph. The interpretation is obvious. Group *a* contains the

fibers that had remained unaffected by the operation; group *b* consists of fibers that had lost their innervation and not regained it; while fibers of group *c* had lost their original innervation and begun to atrophy, but then had intercepted new innervation and thus cut short further atrophy. The earlier this occurred, the less atrophy and the more time there has been for recovery. Therefore, some of the fibers now in class *a* are presumably former *c* members. Of the eighty-seven fibers of the four sample fascicles, sixty-three (i.e., 72 per cent) lie within the normal size range. For the whole muscle, the figure is slightly lower, namely, 65 per cent. Actually, the proportion of innervated muscle fibers is even higher, since as one can readily see from the graph, some of the fibers below 15 micra range definitely with the group of recovering, rather than of denervated, fibers.³

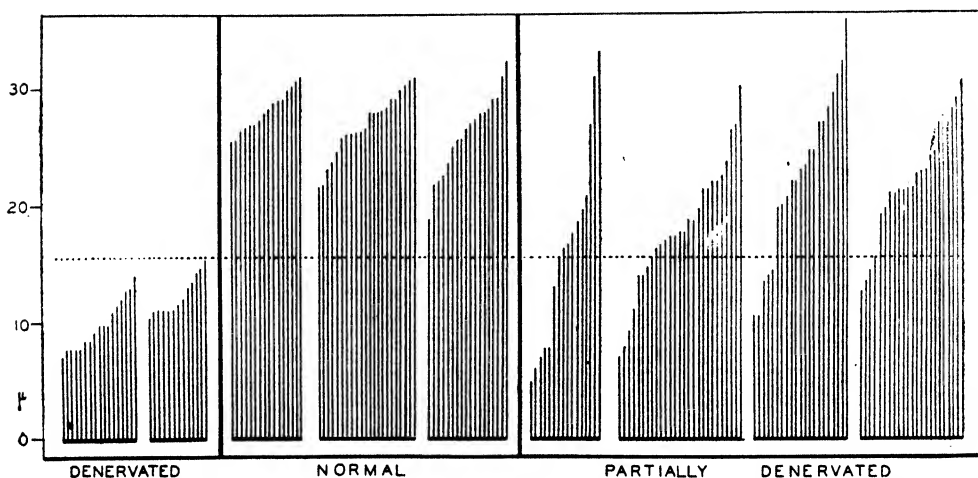


Fig. 3. Muscle fiber sizes (diameter) in sample fascicles from normal soleus muscle and from soleus muscles totally and partially denervated for 44 days (R654).

Now, the nerve of this muscle, by actual count (table 3, R654), contained only six large and thirteen small medullated fibers, which is less than 20 per cent of the normal quota. Thus, while at least 80 per cent of the muscle fibers were initially denervated, less than 35 per cent are still in this class after 44 days. This shows the rapid rate at which the denervated fibers pick up innervation. As a result, each residual motoneuron has become connected with an average of three to four times as many muscle fibers as it had originally supplied. The actual nerve fiber to muscle fiber ratios in this case were approximately 1:15 for

³The figures reported here for histological recovery seem to conflict with the apparent full weight recovery of this muscle (R654) indicated in table 1. However, since the control muscle in this case was of abnormally low weight (table 4, column *a*), the experimental muscle, which had reached the same weight as its control (100 per cent, table 1), actually is still considerably short of its full normal weight.

the control and 1:60 for the experimental muscle. However, because the nerve count includes a few proprioceptive fibers, the actual size of the motor units is somewhat larger than is indicated by these figures.

These data make it plain that the compensatory reaction is due to prompt reinnervation of some of the denervated muscle fibers. Since the number of nerve fibers just proximal to the muscle has not materially increased at the time when the compensatory reaction is already well advanced, the source of this compensatory innervation must be sought in intramuscular nerve sprouts. Later, extramuscular sprouts of the origin described above (p. 593) add to the effect, as is illustrated by the following case, of which sample fascicles are shown in figure 4.

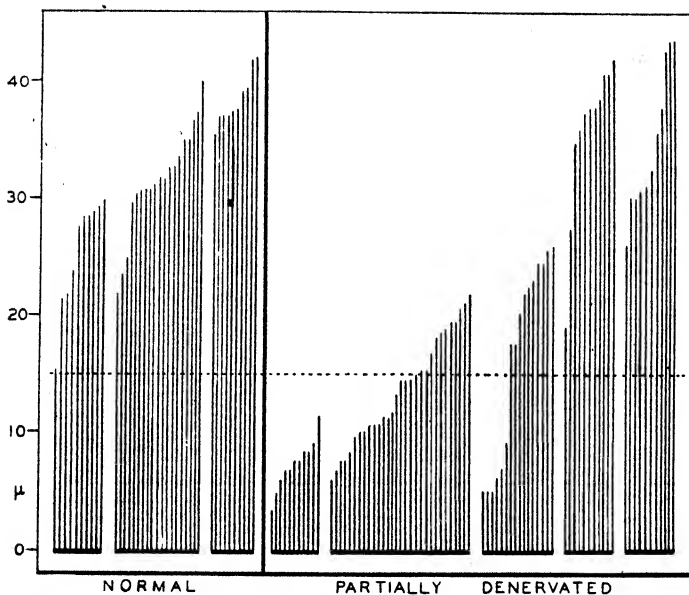


Fig. 4. Muscle fiber sizes in sample fascicles from a normal and partially denervated (R669) soleus muscle, 102 days p. op.

In animal R669, sacrificed 103 days after elimination of L5, the soleus nerve contained 45 per cent of its normal quota (40 per cent of them regenerated branches of small caliber; table 3). These have innervated 80 per cent of the muscle fibers, that is, about double their normal load. In contrast to the previous case, R654, many fascicles contain only fibers of the *b* and *c* types, but none of class *a*. These are, therefore, fascicles that had first undergone complete atrophy, but eventually regained some nerve connections. As the size of their fibers indicates (see graph), they are still in relatively early stages of recovery. This corroborates the assumption that they have received their innervation from nerve fiber branches that had arisen at levels higher up in the nerve and had not reached the muscle until the end of the second month.

By comparing these two cases (R654 and R669), the relative contributions of intramuscular and extramuscular branching to the compensatory reaction can be roughly estimated. In R654, after about 6 weeks, as many as 57 per cent of the originally denervated fibers were already reconnected with intramuscular sprouts. In R669, after an additional 8 weeks, 75 per cent of the denervated fibers had received reinnervation, which is an increase of only about another 18 per cent. Therefore, even if this whole later recovery were to be credited to the forty-odd extramuscular branches of late arrival (table 3), it still would have had only about one-third the effectiveness of the earlier intramuscular expansion. It thus appears that the rate of reinnervation is merely a matter of opportunity. Since the chances for nerve sprouts to encounter uninervated muscle fibers decline as reinnervation progresses, it is to be expected that the rate of recovery will show a corresponding decline with time. Eventually, a great majority of the muscle fibers may receive reinnervation and recuperate. Thus, in our oldest case, R652, 118 days p. op., only about 5 per cent of the total fibers gave the histological appearance of being completely atrophied, i.e., still denervated. Of the reinnervated fibers, many were still of reduced sizes (i.e., on the way to recovery), which explains the fact that muscle strength at this stage was still deficient (table 6).

The results here reported for soleus muscles deprived of L5 are confirmed by the reciprocal cases lacking L4, 6. Only because of the smaller size of the latter contribution, the original deficiency was less marked and the compensatory recovery quicker. Muscles examined 6 weeks after the operation or later rarely contained any atrophying fibers. This histological finding is in full agreement with the observation that these muscles had completely recovered their normal weights, and even slightly overshot their controls (table 1).

In contrast to the clear proof of compensatory innervation, we have found no evidence of appreciable hypertrophy of fibers. Such hypertrophy could manifest itself either in a uniform enlargement of all residual innervated fibers, raising the size maximum, or in an increase of the smaller fibers only, without exceeding the normal upper size limit. Our preparations have revealed no certain signs of either. The largest fibers of the various control soleus muscles measured 45 micra, and none of the fibers of the experimental muscles exceeded this limit. The largest fibers of figures 3 and 4 happen to be slightly larger in the partially denervated than in the control muscles, but this difference does not apply generally. Moreover, the partially denervated fascicles contain decidedly fewer, not more, fibers of sizes close to the upper limit.

In this connection, we call attention to a technical source of deception in deducing fiber hypertrophy from cross sections. Some of our preparations contained fibers with greatly oversized cross sections, suggestive of hypertrophy. They appeared in both control and partially denervated muscles in variable numbers, measured up to 100 micra across, and were circular in outline. Closer study revealed an artifact, resulting from excessive retraction in the fixing fluid of occasional loose fibers in an otherwise stretched muscle. Wohlfart (1937) has described fibers of this type as "Kontraktionsknoten," and emphasized their deceptiveness. For a crucial test, we made a small cut across the posterior

border of a soleus muscle a few millimeters proximal to the tendon of insertion, and then stretched and fixed the muscle in the usual way. Cross sections through the middle showed that the muscle fibers which had not been held under tension during fixation all had excessively large diameters.

DISCUSSION. The reported experiments have 1, established the fact of "spontaneous" recovery of partially denervated muscle, referred to above as "compensatory reaction"; and 2, produced evidence concerning its mechanism. By the combined use of several criteria and methods of examination—muscle weights; isometric tensions; nerve counts; muscle histology—, a fully consistent concept of the events following partial denervation has been obtained.

Partial denervation was achieved by severing some of the roots of nerves of plurisegmental origin. The standard reduction of nerve supply thereby produced was determined and its permanency insured by effective occlusion of the severed roots. Nerve regeneration in the ordinary sense, namely, from the cut ends of the proximal nerve stumps, was thus excluded. After the operation, the nerves consisted of intact and interrupted fibers in various known proportions. The interrupted fibers underwent Wallerian degeneration, and the muscle became composed of two kinds of fibers, innervated and denervated ones, in proportions reflecting the composition of its nerve. If the intact muscle units behaved as they would in normal muscle, and the denervated ones as they would after total denervation, there should follow a period of progressive atrophy, reducing the muscles to sizes and strengths in proportion to the initial innervation deficit. This did not happen.

We have no data on the events during the first week; nor do we consider the deficits in our only two 8-day cases (tables 2, 4, 5, 6) as typical. From the second week on, however, the trend is clear. All muscles after that period exhibit greater weight and strength than could be accounted for by adding up intact and denervated parts in their original proportions. The partially denervated muscle, taken as a whole, not only loses less weight, and more slowly, than would be commensurate to its loss of innervation, but the losing trend is gradually reversed and the muscle regains part or all of its previously lost weight and strength (tables 1, 2, 5, 6).

In explanation of this "compensatory reaction", we first looked for hypertrophy of the unharmed muscle fibers, but our histological studies have failed to substantiate it. In human pathology, on the other hand, hypertrophy of individual fibers has been noted in cases of progressive muscular dystrophies (e.g., amyotrophic lateral sclerosis; Pilez, 1898, and others), but according to the careful studies of the Wohlfarts (1935), the occurrence is sporadic and the degree rather slight (see also Karlström and Wohlfart, 1939). However, these cases and our own experiments are not strictly comparable, because of the great difference between human and rat muscles in regard to load and functional stress, which predisposes the former to a wider range of "functional" hypertrophy.

All our observations concur in proving that the "compensatory reaction" is due chiefly to reinnervation of the denervated muscle fibers. Since the nerve fibers severed in the operation have been blocked from growing back into the muscle, a spontaneous peripheral amplification by branching of the residual

intact fibers seems to have taken place; "spontaneous" insofar as the production of new branches did not start from the site of the operative lesion. A similar "spontaneous" extension of collateral nerve branches into a denervated area has been observed in the skin (Speidel, 1940; Weddell, Guttman and Gutmann, 1941), but the precise mechanism of this phenomenon has remained obscure (see Weiss, 1941). Our present experiments contain at least certain positive clues. The problem poses three main questions: 1. Where are the new nerve branches formed, and as a result of what stimulus? 2. Do the mother fibers, from which they sprout, retain their peripheral connections intact (collateral regeneration), or do they first undergo some degeneration, followed by regeneration with terminal branching? 3. Are the new sprouts actively drawn towards the denervated muscle fibers, or do they simply roam and make connections by accident?

1. *Source of branches.* It seems an established fact that a single muscle fiber rarely accepts more than one nerve ending (Elsberg, 1917; Fort, 1940). Though immune to supernumerary innervation during its innervated state, the muscle fiber becomes receptive again within several days after the loss of its innervation (Fort, 1940). In view of this situation, it is conceivable that muscle normally contains a surplus of terminal nerve fiber branches which have failed to connect and are functionally idle. Wholly unconnected nerve fibers certainly persist without resorption (Weiss and Taylor, 1944; Weiss and Edds, 1945; Weiss, Edds and Cavanaugh, 1945), and so do unattached terminal branches (Gutmann and Young, 1944). If such terminal fiber reservoirs exist, they could be claimed as source of the "compensatory reaction." There is at present no definite proof either for or against such an assumption, but if the reservoirs were so abundant as to account for our results, it is doubtful that they could have escaped notice. It is more likely that the excess branches in our experiments were newly formed after the operation in response to a stimulus vaguely identifiable with some "traumatic" or "irritant" action of the degenerating nerve branches and their denervated end-organs.

The nature of this action remains to be investigated. It could either be a mass effect of chemical products diffusing from an injured region and "irritating" healthy axon branches, even at some distance, or it could be simply a local effect exerted by a degenerating nerve fiber or denervated muscle fiber upon any healthy axon in immediate contact with it. Our experiments favor the latter view for the following reasons. Innervated and denervated units in our experimental muscles are not cleanly segregated but lie interspersed with each other. Contrary to a common notion, the individual fascicle does not correspond to a single motor unit, that is, the muscle fibers of any given fascicle may receive innervation from more than one motoneuron, and branches of any given motoneuron may terminate in more than one fascicle (Wohlfart, 1935). This is confirmed by the fact that many fascicles of our partially denervated muscles contain both innervated and denervated fibers (fig. 3). There is thus ample opportunity for direct contact between surviving nerve branches and denervated elements.

In order to test experimentally whether such intimacy of contact between

defective and intact units was essential for the "compensatory reaction", we chose a muscle preparation in which denervated and innervated portions could be obtained in strict segregation, namely, the diaphragm. The two halves are partly separated by a tendinous inscription, but there is a triangular area ventrally where there is no mechanical barrier to oppose the passage of nerve fibers from one half to the other. Normally there is no overlap of innervation between the two halves. We cut the phrenic nerve on one side in three animals. Histological examination 3 weeks afterwards revealed that no nerve branches had crossed over the midline from the intact side to take over the denervated portion. Clearly, therefore, denervated muscle fibers, when lying in a solid and sharply delimited block free of intact axons, do not evoke the compensatory axon branching which they evoke when interspersed with innervated fibers.

The same seems to apply to "spontaneous" branching within the nerve itself. As reported above, the surviving axons of the partially denervated nerve trunks proliferate branches. Counts of old and regenerated fibers present in the soleus nerve at various periods after partial denervation (table 3) revealed that for some time after the operation, individual old fibers tend to break down at some peripheral point and then regenerate with one or two dichotomies. This delayed axon disruption may be a result of the local pressure exerted by the enormous swelling of surrounding fibers undergoing Wallerian degeneration. Such a compression of intraneural origin would have the same disruptive effect on axons as does external constriction (Cajal, 1928; Weiss and Davis, 1943; Denny-Brown and Brenner, 1944).

(2). *Collateral or terminal regeneration?* The fact that the appearance of regenerating branches is preceded by the disappearance of old fibers (table 3) is proof that the former do not arise as collaterals from intact fiber stems, but by the standard process of terminal regeneration following breakdown of the distal portion of the original fiber. Presumably the same happens in intramuscular branching; that is, prior to its compensatory proliferation, a nerve fiber branch would first have to lose temporarily its old peripheral connections. The collateral sprouting of cutaneous fibers observed by Speidel (1942) to occur near wounds in frog larvae, may no longer be possible in mature fibers possessing firm neurilemmal and myelin sheaths.

As in the case of intramuscular proliferation, so the compensatory branching inside the nerve seems to occur only if intact and degenerating units lie intermingled. This is the case after section of segmental roots. If, on the other hand, a partial lesion is made in a peripheral nerve trunk distal to the plexus, so that intact and degenerating portions remain in separate groups, the intact fibers retain their integrity and no compensatory regeneration occurs (Lugaro, 1906). As Lugaro has correctly inferred, there is no "neurotropic" stimulus of the degenerated half that would invite the fibers of the intact half to send forth collateral sprouts; only actual physical disruption can initiate regeneration in the mature axon. The concept of "neurotropism" thus becomes just as untenable as a factor of fiber proliferation as it is invalid in fiber orientation (Weiss and Taylor, 1944).

3. *Neuro-muscular connections.* In view of the persistent failure to detect any sign of positive "neurotropic attraction" of nerve branches toward distant destinations, the extension of the new nerve fiber branches over the denervated muscle fibers must be viewed as a matter of chance. Since muscle fibers can be reinnervated either through the old end-plate or at a new site (Fort, 1940; Gutmann and Young, 1944), the new branches need not even follow the old pathways, but may simply attach themselves to any uninnervated muscle fibers that lie across their path. With the progress of reinnervation, the chances of roaming branches to encounter still uninnervated muscle units decline. This explains why the compensatory reaction is much more spectacular during the earlier than during the later stages (see p. 601). It is even likely that some of the late coming branches, which have regenerated from high up in the nerve, fail to find unoccupied muscle fibers; this is indicated by the unusually high quota of small fibers in the nerve even after 4 months (R652, table 3), for unconnected fibers always remain small (Weiss, Edds and Cavanaugh, 1945; Sanders and Young, 1945).

The reported facts suggest that clinically, a similar "spontaneous" improvement in the muscular condition could be expected after any partial nerve lesion in which the involved axons are scattered among healthy axons. This is true of partial poliomyelitic lesions, and it would seem indicated to examine whether a major share of the gradual gain in muscle strength after partial paralysis might not be attributable to this source. Actually, the body produces here automatically what Dogliotti (1935) and Billig and van Harreveld (1943) have proposed to enforce surgically, namely, a compensatory peripheral amplification of the reduced nerve source. In view of this fact, the surgical procedure of nerve crushing advocated by Billig (1944) would seem to be not only risky, but superfluous. On the other hand, partial severance of a peripheral nerve trunk, leaving degenerated and healthy portions segregated, cannot be expected to result in a marked compensatory reaction except in the rather narrow zone of overlap along the boundary between the denervated and intact areas.

SUMMARY

Incomplete denervation of muscles in the rat was found to be followed by "spontaneous" recovery not attributable to regeneration of the severed nerve fibers. This recovery was studied quantitatively by following the weight, strength and histological changes in the muscles and comparing them with the residual nerve supply.

Partial denervation was effected by cutting the spinal nerves L4 and 6 or L5. The proximal stumps were prevented from regenerating by capping. Weight changes were recorded for six major leg muscles (tables 1, 2). For a more detailed study the soleus muscle was selected (table 4, 5).

From the knowledge of the size of the denervated fraction and of the amount of atrophy following total denervation, the expected weights of muscles partially denervated for various periods could be computed. Except for the first week, the weights actually observed were much higher than calculated, that is, the

denervated portion of a partially intact muscle atrophies less than it would if the muscle were completely denervated. Within a few weeks, atrophy is checked and the muscle gradually returns to nearly its original size.

Isometric tensions after stimulation of the nerve show a trend roughly parallel to the weight changes (table 6).

Histological study showed a progressively increasing number of muscle fibers recovering normal size after some initial atrophy. Four months after the denervation of 80 per cent of the muscle fibers, only 5 per cent were still in an atrophic (i.e., denervated) state. At no time was there evidence of hypertrophied fibers.

All observed facts lead to the conclusion that the intramuscular branches of the intact motor nerve fibers undergo additional branching and take over the supply of the denervated muscle fibers. This occurs only when intact and denervated elements lie intermingled; the stimulus for branching lies presumably in some traumatic contact action, and no "neurotropic" stimulation of branching or "attraction" of branches toward a solid denervated area has been noted.

A similar phenomenon was discovered in the nerve trunk, where some of the fibers which had not been severed in the operation, later broke down "spontaneously" and then regenerated with some branching. This occurrence, too, is conditional on the diffuse dispersal of intact among degenerating nerve fibers, and the pressure of the latter may be assumed to furnish the traumatic stimulus.

The results suggest that similar compensatory branching with consequent improvement of muscle weight and strength might occur after diffuse poliomyelitic lesions, but not after partial traumatic injury.

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Addendum to proofs: After the completion of the work described in this article, a paper reporting similar results was published by A. van Harreveld (This Journal **144**: 477, 1945), too late for inclusion in the discussion.

EFFECT OF SOME ISOCYCLIC, AROMATIC AND HETEROCYCLIC COMPOUNDS ON MUSCLE SENSITIVITY TO ACETYLCHOLINE AND POTASSIUM¹

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Several substances are known to modify the sensitivity of effector cells to acetylcholine independently of their effect on the activity of cholinesterase (1, 2) (alcohols (3, 4), urethanes (3, 4), chloroform (5), ether (5), physostigmine (2, 6, 7), some organic phosphates (8), vitamin B₁ (9)). The effect of alcohols and urethanes was explained by their ability to lower the surface tension (4, 10). In the following, the effect of some aromatic hydrocarbons and heterocyclic compounds on the acetylcholine and potassium sensitivity of striated muscle was investigated. Some of these substances decrease the surface tension to a degree comparable to that of the alcohols.

EXPERIMENTAL. I. *Effect of the substances in inducing shortening of muscle.* The rectus abdominis muscle of the frog was excised and suspended in a muscle chamber containing 10 cc. Ringer's solution. The Ringer's solution was changed to a solution of the substances used for 5 minutes. The muscle was then washed for 10 minutes with Ringer's solution. This procedure was repeated, increasing the concentration of the substances. The pH of all solutions used here, and in the following experiments, was corrected to 7. The height of shortening of the muscle was registered by an isotonic lever on a kymograph. Whenever a shortening occurred as a result of immersion in one of the solutions the letter "s" was inserted in the table.

The results are given in table 1. Within the concentrations used, benzol, acetophenone, benzaldehyde, phenol, hydroquinone, α -naphthol, β -naphthol, indol and quinoline induced a shortening of the rectus abdominis muscle. The amounts of benzol and quinoline required to induce shortening of the muscle was one tenth of the amount required of the other substances.

II. *Effect of the substances on acetylcholine sensitivity of striated muscle.* The rectus abdominis muscle was prepared as described above. Shortening of the muscle was induced throughout the entire experimental procedure by immersion in an acetylcholine solution (50 μ g per 100 cc. Ringer's solution) for two minutes. Between two immersions in acetylcholine the muscle was washed with Ringer's solution for ten minutes. This procedure was repeated until three successive immersions in acetylcholine gave similar responses. Then between two shortenings induced by acetylcholine instead of washing in Ringer's solution for ten minutes, the muscle was washed only for five minutes and immersed in one of the solutions to be tested for five minutes. A series of solutions containing the substances in increasing concentrations was used. The amount of muscle

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shortening was registered by an isotonic lever on a kymograph, and the tracings were measured. The amount of shortening of the muscle after immersion in the solutions of the substances was expressed as percentage of the amount of shortening of the same muscle before immersion in the solutions. The amount of shortening induced by acetylcholine in control muscles immersed only in Ringer's solution remained unchanged for at least three hours. This period of time was longer than the duration of the experiments described. Since the S. E. of the mean for each experiment was less than ± 4 per cent, all results deviating from 100 per cent by more than 12 per cent are probably significant deviations ($2\sqrt{S.E.^2_{(control)} + S.E.^2_{(experiments)}} = 2\sqrt{4^2 + 4^2} = 11.5$).

The effect of the substances on the acetylcholine sensitivity are given in table 1. The substances that induced shortening of muscle, as described above, increased the sensitivity of muscle to acetylcholine in lower concentrations. The acetylcholine sensitivity was also modified by some of the other substances. Benzol and quinoline were the most effective sensitizing agents. Saturated compounds (cyclohexane) were much less effective in increasing the acetylcholine sensitivity of the muscle than unsaturated ones (benzol, etc.). Introduction of one or more hydroxyl groups modified the activity of the different compounds in different ways: 1, the sensitizing effect decreased (benzol vs. phenol; phenol vs. hydroquinone; cyclohexanol vs. inositol; quinoline vs. 8-hydroxyquinoline); 2, the sensitizing effect was not modified (benzoic acid vs. salicylic acid); 3, the sensitizing effect increased (toluol, benzoic acid vs. benzyl alcohol; naphthalene vs. α -naphthol, β -naphthol). Increasing substitution of the aromatic ring with certain groups decreased the sensitizing effect of the compounds (benzol vs. toluol, aniline, benzoic acid, p-amino benzoic acid, m-dinitrobenzene; aniline vs. sulfonamides; phenol vs. potassium phenol sulfonate, p-amino phenol; the sensitizing effect gradually decreased by substitution in the following series: indol, skatol, indol-3-acetic acid, l(-)-tryptophane). Acetylation also decreased the sensitizing effect of the substances (aniline vs. acetanilide; salicylic acid vs. acetylsalicylic acid). Oxidation of the alcohols to aldehyde increased the sensitizing effect of the substances (benzyl alcohol vs. benzaldehyde). The structurally similar ketone (acetophenone) had approximately the same effect as the aldehyde (benzaldehyde).

To ascertain whether the increase of sensitivity to acetylcholine was due to the mechanism responsible for the sensitization of muscle by physostigmine, muscles were immersed for one hour in Ringer's solution containing 2.5 mgm. physostigmine salicylate per 100 cc. The substances used were dissolved in physostigmine solutions, and the experiments were carried out as described above, except that the physostigmine solutions were used instead of Ringer's solutions. Shortening of the muscle was induced by a solution containing 10 μ g acetylcholine per 100 cc. Ringer's solution. (The physostigmine solution was concentrated enough to cause a nearly maximal sensitization of the muscle to acetylcholine.) The results are given in table 1. The sensitivity of the eserinated muscle to acetylcholine was increased by the substances used. This increase, however, was less than the increase of the acetylcholine sensitivity of the non eserinated muscles, suggesting that the sensitization by the cyclic compounds

TABLE 1

Effect of the substances on the shortening of the rectus abdominis muscle

SUBSTANCE	MAGNITUDE OF SHORTENING IN % OF CONTROL.* SHORTENING INDUCED WITH:																	
	Acetylcholine												Potassium					
	(Non eserized muscle)						(Eserized muscle)											
	Concentrations of the substances (mgm) per 100 cc Ringer's solution:																	
	100	10	1	0.1	0.01	0.001	100	10	1	0.1	0.01	0.001	100	10	1	0.1	0.01	0.001
Isocyclic hydrocarbons:																		
Cyclohexane.....	116	110	99	100	100	101							389	285	164	115	95	103
Hydroxyl derivatives:																		
Cyclohexanol.....	248	145	117	108	102	100							196	140	125	119	113	105
Inositol.....	110	103	104	103	95	98							133	132	117	111	109	101
Aromatic hydrocarbons:																		
Benzol.....		s†	228	115	104	100		s	151					s	208	118	97	94
Toluol.....	106	103	96	96	98	100							156	133	120	116	109	104
Naphthalene.....		110	113	109	106	104								223	159	142	127	103
Aromatic carboxylic acids:																		
Benzoic acid.....	118	111	103	100	101	100							144	160	144	125	120	111
Aromatic alcohols:																		
Benzyl alcohol.....	292	142	101	100	102	100							211	191	166	139	118	109
Aromatic ketones:																		
Acetophenone.....	s	171	104	99	101	100							s	66	88	116	113	105
Aromatic aldehydes:																		
Benzaldehyde.....	s	166	112	105	99	101	s	134	105	100	103	99	s	70	105	105	112	107
Hydroxyl derivatives:																		
Phenol.....	s	238	141	107	102	105	s	150	120	104	101	106	s	242	175	138	130	128
Hydroquinone.....	s	185	105	102	99	101							s	69	94	97	98	99
Salicylic acid.....	102	120	101	100	101	99							142	192	123	117	103	102
α-Naphthol.....	s	208	124	105	102	100							s	365	191	137	129	103
β-Naphthol.....	s	191	127	114	105	103							s	393	200	147	118	100
8-Hydroxyquinoline...	24	96	93	97	100	100							152	143	139	134	110	100
Conjugated compounds:																		
p-Amino benzoic acid.	98	100	104	104	105	102							107	116	115	120	114	103
m-Dinitrobenzene.....	164	123	104	101	98	100	126	117	98	103	100	102	255	157	115	110	107	106
Potassium phenol sulfonate.....		106	110	108	101	102								117	109	110	104	101
p-Amino phenol.....	171	111	97	96	97	100							122	144	148	130	131	119
Diphenylamine.....	116	112	100	102	105	103							455	316	167	147	130	122
Aniline.....	565	258	118	104	102	100							231	166	161	124	112	100
Benzidine.....	157	105	98	102	102	101							55	55	69	88	100	98
Acetanilide.....	210	110	98	99	100	101							166	111	100	102	98	101
Sulfanilamide.....	113	102	100	99	100	100							63	86	108	101	100	100
Sulfapyridine.....	123	102	102	99	98	101							66	79	102	102	100	99
Sulfathiazole.....	139	111	100	103	101	102							50	76	93	100	102	100
Sulfadiazine.....	116	100	98	97	99	100							69	78	100	99	99	100
Acetylsalicylic acid...	94	98	102	106	99	98							101	107	108	109	106	103

TABLE 1—*Concluded*

SUBSTANCE	MAGNITUDE OF SHORTENING IN % OF CONTROL.* SHORTENING INDUCED WITH:																	
	Acetylcholine												Potassium					
	(Non eseriniz muscle)						(Eseriniz muscle)											
	Concentrations of the substances (mgm) per 100 cc Ringer's solution:																	
	100	10	1	0.1	0.01	0.001	100	10	1	0.1	0.01	0.001	100	10	1	0.1	0.01	0.001
Heterocyclic compounds:																		
Indol.....	s	224	114	108	101	102	s	135	101	102	99	100	s	230	126	115	112	110
Skatol.....	102	97	95	100	98	98							535	300	132	120	107	105
Indol-3-acetic acid.....	110	96	97	98	101	98							200	153	130	125	117	103
l(-)tryptophane.....	117	108	102	103	101	99							102	110	109	104	105	100
Quinoline.....	s	238	133	107	99		s	139	117	110	100		s	128	112	98	101	
Carbazole.....	105	106	100	97	100	101							118	121	116	115	114	100
Oleic acid.....	86	88	93	95	98	100							136	119	122	123	103	98

* Each value represents the average of 10 separate experiments. The S.E. of the mean for each value was less than $\pm 4\%$.

† "s" means that muscle shortening occurred during the immersion of the muscle in the solution for 5 minutes without addition of acetylcholine and potassium.

was caused by at least two mechanisms: 1, inhibition of the activity of cholinesterase, and 2, another not yet identified mechanism.

III. *Effect of the substances on potassium sensitivity of striated muscle.* Acetylcholine sensitivity usually parallels the sensitivity of the muscle to indirect stimulation. To ascertain whether the substances modify the sensitivity of muscle to other chemical stimuli, shortening of the muscle was induced by a 20 mM KCl solution instead of acetylcholine, and the effect of the substances on the potassium sensitivity of the muscle was observed as described above. The potassium sensitivity of muscle often parallels the effect of direct stimulation of the muscle (11).

The results are given in table 1. Unsaturated products seemed to have a greater sensitizing effect than saturated ones (benzol vs. cyclohexane). Introduction of one or more hydroxyl groups modified the activity of the different compounds in different ways: 1, the sensitizing effect decreased (cyclohexane vs. cyclohexanol; cyclohexanol vs. inositol; benzol vs. phenol; phenol vs. hydroquinone); 2, the sensitizing effect was not modified (benzoic acid vs. salicylic acid); 3, the sensitizing effect increased (naphthalene vs. α -naphthol, β -naphthol; toluol, benzoic acid vs. benzyl alcohol). Increasing substitution of the aromatic ring with certain groups decreased the sensitizing effect of the compounds (benzol vs. toluol, benzoic acid, aniline, m-dinitrobenzene; aniline vs. sulfonamides; benzoic acid vs. p-amino benzoic acid; phenol vs. potassium phenol sulfonate, p-amino phenol; indol, skatol vs. indol-3-acetic acid, l(-)-tryptophane). Acetylation also decreased the sensitizing effect of the compounds (aniline vs. acetanilide; salicylic acid vs. acetylsalicylic acid). Oxidation of alcohols to aldehyde

did not increase the sensitizing effect (benzyl alcohol vs. benzaldehyde). The structurally similar ketone (acetophenone) had approximately the same effect as the aldehyde (benzaldehyde).

Most of the substances increased the sensitivity of muscle to potassium, suggesting that the absence of changes in acetylcholine sensitivity was not due to the low solubility of the substances used. Changes of potassium sensitivity often did not parallel the changes of acetylcholine sensitivity, suggesting that sensitization to acetylcholine and potassium do not depend on exactly the same mechanisms.

DISCUSSION. The results presented above suggest that some of the substances used modify the acetylcholine sensitivity of striated muscle partly by the same mechanism as physostigmine and partly by other mechanisms. Sensitization to acetylcholine and potassium seem to depend, at least partly, on different mechanisms.

Meng (4) found the following relationship between physicochemical properties of the members of the alcohol series and their effect on the acetylcholine sen-

TABLE 2
*Interfacial tension between the substances and water**

SUBSTANCE	INTERFACIAL TENSION
Benzol.....	35.00
Toluol.....	36.10
Cyclohexanol.....	3.92
Benzyl alcohol.....	4.75
Benzaldehyde.....	15.51
Aniline.....	5.77
Oleic acid.....	15.59

* From the International Critical Tables, McGraw-Hill, Inc., New York, 1928, Vol. 4, p. 436-437.

sitivity of striated muscle: 1, the threshold concentrations inducing shortening of muscle are smaller with the substances producing a greater increase of acetylcholine sensitivity; 2, introduction of a hydroxyl group decreases the sensitizing effect of the alkyl radical, and introduction of a second or third hydroxyl group decreases the sensitizing effect to an even greater degree; 3, the sensitizing effect of the alcohols increases upon elimination of the hydroxyl group (oxidation to aldehyde or ketone); 4, desaturation of the alcohols brings about a decrease in the sensitizing effect; and 5, the surface activity of the alcohols parallels their sensitizing effect.

A similar parallelism could not be established with the substances used in our experiments. 1, The sensitivity of muscle to acetylcholine and potassium was usually increased with concentrations approximating those inducing shortening of muscle. Also, the lower the concentrations required to induce shortening of muscle, the lower the concentrations that sensitized the muscle to acetylcholine and potassium; 2, introduction of a hydroxyl group may either decrease or increase the sensitizing effect of the substances or leave it unaltered; 3, the acetyl-

choline sensitivity of the muscle was greater in the presence of benzaldehyde and acetophenone than that of benzyl alcohol; the potassium sensitivity of muscle was more in the presence of benzyl alcohol than that of benzaldehyde and acetophenone; 4, unsaturated compounds had a greater sensitizing effect than the saturated compounds; 5, a parallelism between surface activity and effect on acetylcholine sensitivity could not be established. The interfacial tensions between water and some of the substances are given in table 2. The interfacial tensions between toluol and water and benzol and water are similar, but benzol induced a shortening of the muscle and increased acetylcholine sensitivity while toluol was ineffective. The interfacial tension between benzaldehyde and water is lower than between water and benzol, but benzaldehyde was less effective in our experiments. The interfacial tension between water and oleic acid is about the same as between water and benzaldehyde, but oleic acid seemed to be nearly inactive in our experiments. The interfacial tensions between benzyl alcohol, aniline, cyclohexanol and water are the smallest, but these substances did not induce shortening of muscle and increased the acetylcholine sensitivity of muscle only to a moderate degree. Aniline increased the effect of acetylcholine twice as much as did cyclohexanol and benzyl alcohol. The effect of the substances on the potassium sensitivity of the muscle did not parallel the surface activity of the substances either. It is likely that changes of the surface tension will induce changes leading finally to changes of the shortening of the muscle. The above experiments suggest, however, that the substances modified not only the surface tension of the muscle cells, but also induced chemical changes in the muscle.

SUMMARY

1. The effect of some cyclic compounds in concentrations from about 10^{-2} to 10^{-7} mol. on the rectus abdominis muscle was investigated.

2. Shortening of the muscle was induced by benzol, phenol, hydroquinone, benzaldehyde, acetophenone, α -naphthol, β -naphthol, indol and quinoline. Benzol and quinoline were the most effective agents.

3. The acetylcholine sensitivity of muscle was increased by cyclohexanol, benzol, benzyl alcohol, acetophenone, benzaldehyde, phenol, hydroquinone, α -naphthol, β -naphthol, m-dinitrobenzene, p-amino phenol, aniline, benzidine, acetanilide, sulfonamides, indol, quinoline, and to a small extent by benzoic acid, salicylic acid, cyclohexane, inositol, naphthalene, diphenylamine, and l(-)tryptophane.

4. The acetylcholine sensitivity was not modified by toluol, p-amino benzoic acid, potassium phenol sulfonate, acetylsalicylic acid, skatol, indol-3-acetic acid, carbazole.

5. The acetylcholine sensitivity was decreased by 8-hydroxyquinoline and oleic acid.

6. The potassium sensitivity was decreased in the presence of acetophenone, benzaldehyde, hydroquinone, benzidine, and the sulfonamides.

7. The potassium sensitivity was not modified by acetylsalicylic acid, and l(-)tryptophane and was increased by the other substances used.

8. The increase of the acetylcholine sensitivity was caused by both a physostigmine sensitive and another mechanism. Changes of acetylcholine sensitivity often did not parallel the changes of potassium sensitivity suggesting that acetylcholine and potassium sensitivity depend, at least partly, on different mechanisms. Shortening of muscle and changes of the sensitivity of muscle to chemical stimuli were probably due not only to the surface activity of the substances but also to as yet unidentified chemical mechanisms.

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THE CONFIGURATION OF EPICARDIAL AND ENDOCARDIAL EXTRASYSTOLES IN THE CHEST LEADS¹

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The supposition that the QRS complex of the electrocardiogram as derived from the standard limb and chest leads records the electrical concomitants of the travel of the cardiac impulse from its first appearance in the endocardium of the interventricular septum to the moment of final activation of the entire heart when the base of the left ventricle, or the conus of the right ventricle is excited, finds its main support, insofar as experiments employing indirect leads in the mammal are concerned, in experiments reported by Lewis (1). In four experiments he recorded the electrocardiogram from a transthoracic lead and elicited extrasystoles from two points facing each other on the endocardium and epicardium of the right ventricle. Lewis excused the fact that two points only were stimulated by suggesting that moving the point of stimulation a few millimeters could hardly be expected to make any difference in the record obtained. When the electrocardiograms of the resulting extrasystole were examined, it was found that the epicardial extrasystole showed only an R wave, while in the endocardial extrasystole a Q wave preceded R. Lewis assumed that this Q wave represented the outward travel of the wave of excitation, which would be of opposite polarity to the inwardly travelling wave of the epicardial extrasystole, as can best be illustrated by recourse to the dipole notational system.

With this evidence that an impulse travelling within the endocardium can influence the indirect leads, the theory has been extended to the standard limb leads (2) and to the chest leads (3) without further examination of the experimental evidence. As far as the chest leads are concerned, the supposition is made that the entire rising phase of the R wave represents the outward travel of the impulse from endocardium to epicardium in the region under the chest electrode, while the downstroke of R occurs as the impulse breaks through the epicardium in that area.

To this hypothesis certain objections may be raised. It is apparent that according to it a given wave can be identified only by reference to a hypothetical normal sequence of excitation. In the presence of altered conduction, or when faced by ventricular extrasystoles of unknown origin, so many possibilities exist that localization becomes impossible. Thus in the chest lead, an R wave might be the sign of an outwardly travelling wave under the electrode, an inwardly travelling wave in the same ventricle some distance away from the electrode, or an impulse reaching the ventricle under the electrode via the interventricular

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septum. A Q wave might indicate an inwardly travelling wave under the chest electrode, or an outwardly travelling wave any other place in the same ventricle. One of the exponents of this view accepts this lack of localizing ability as applied to the T wave when he states that "attempts to determine the metabolic or other effect of certain agents upon the heart muscle from a study of the electrocardiogram of the intact animal is almost futile" (4). This is completely at variance with fact. Extrasystoles may be elicited from all possible regions of the heart, both epicardial and endocardial, but whenever a given wave appears, it can be shown to have the same physiological meaning and anatomical localization as that same wave in any other extrasystole, or for that matter, any normal complex (5). Therefore a Q wave, or an R or S wave, however it may appear in standard limb leads, cannot have a variety of origins, but has only one.

The experiments of Lewis have been re-examined in the standard limb leads, in both right and left ventricles, all possible points on the endocardium and epicardium being stimulated, and it was clearly shown that whatever might be the electrical activity of an outwardly travelling wave of excitation, it could not be detected in the standard limb leads (6). Since, however, the chest leads might be presumed to be more adequately situated either by orientation or sensitivity to record such outwardly travelling waves, and inasmuch as claims are made that waves of the amplitude of R do in fact so arise, the question has been re-examined using a number of the standard chest leads, as well as the transthoracic leads employed by Lewis.

METHODS. Seven dogs were employed, anesthetized with Dial or Nembutal. Epicardial and endocardial extrasystoles were elicited in the right and left ventricles by methods previously described (6). The special electrode holder maintained the internal and external electrodes in apposition across the myocardium, the lungs were fully expanded, the chest opening closed and the animal breathed spontaneously in all instances. Stimuli were delivered at a strength barely above threshold. Four leads were recorded: 1, transthoracic; 2, 4F; 3, 4R and 4, the central terminal lead of Wilson. In all experiments the position of the stimulating electrodes was carefully arranged. In some they were perfectly centered in the line between transthoracic leads or directly beneath the chest electrode, which was shifted from the right to the left side to conform with the ventricle stimulated. In another series of experiments the chest electrode remained over the right or left apex, while the position of the stimulating electrodes was altered and in a final series the stimulating electrodes were kept in a fixed position and the precordial electrode was shifted.

RESULTS. (A) When the stimulating electrodes were carefully aligned with the transthoracic lead, or were immediately underneath the chest electrode in the precordial leads, epicardial and endocardial extrasystoles were indistinguishable, whether elicited from the right or the left ventricle (figs. 1 and 2). This was the case whatever lead was employed. The initial wave of the complex was in every instance a simple downward deflection resembling QS and no sign of an initial upward deflection was ever seen.

(B) When the recording electrodes remained in position and the stimulating

electrodes were moved away from the chest electrode or out of the line between transthoracic electrodes, it was at times possible to record a difference between epicardial and endocardial extrasystoles. Thus in figure 3A no difference between endocardial and epicardial extrasystoles could be detected when the stimulating electrodes were at the right lateral margin of the heart near the apex. When the stimulating electrodes were moved closer to the right base, the endocardial extrasystole was noted to have a small R wave which was lacking in the epicardial extrasystole (fig. 3B). When the stimulating electrodes were subsequently moved still further toward the base, the configuration of epicardial and endocardial extrasystoles was again the same and both now showed an R wave (fig. 3C). Stimulation of more distant regions (fig. 3D) yielded complexes showing only an R wave in both endocardial and epicardial extrasystoles.

(C) Differences in the configuration of epicardial and endocardial extrasystoles could at times be produced by moving the chest electrode, while the stimulating electrodes remained in the same position. In the experiment shown in figure 4, the stimulating electrodes were fixed at the lateral margin of the right ventricle midway between apex and base, while the chest electrode was placed 1, directly external to the stimulating electrodes; 2, anterior to point 1; 3, caudal to point 1 at the lower margin of the thorax; 4, dorsal to the location of the stimulating electrode, as well as 5, as far headward as possible in the axilla. As recorded from points 1, 3 and 5 no differences existed between endocardial and epicardial extrasystoles. In 3 both endocardial and epicardial extrasystoles showed R waves. From points 2 and 4, however, differences were recorded, but it was the epicardial extrasystole that showed a small initial R wave lacking in the endocardial complexes.

(D) At times both epicardial and endocardial extrasystoles showed variability in configuration depending upon the phase of the cycle in which they fell. Usually those occurring early in the cycle showed the simplest form, as can be seen in figure 5. In extrasystoles arising later in diastole, small initial waves opposite in direction to the major initial deflection made their appearance. Thus early extrasystoles showed only a QS, while in extrasystoles arising from the same point but later in diastole an R wave of varying amplitude appeared.

DISCUSSION. Extrasystoles arising from the endocardium of either the right or the left ventricle are indistinguishable from their epicardial counterparts as recorded in the transthoracic lead and leads 4R, 4F and the central terminal lead of Wilson. In this respect these chest leads are no more sensitive than the indirect leads I, II and III. This can only mean that the chest leads, like leads I, II and III, fail to record the potentials that must accompany the outward passage of an impulse from endocardium to epicardium. It is significant that the optimum conditions for revealing such currents would be those in which the stimulating electrodes are directly in the line of transthoracic leads or directly beneath the precordial electrode in leads 4R, 4F and Wilson's lead, and that these are precisely the conditions in which no differences occur between the endocardial and epicardial extrasystoles.

Differences in configuration between endocardial and epicardial extrasystoles

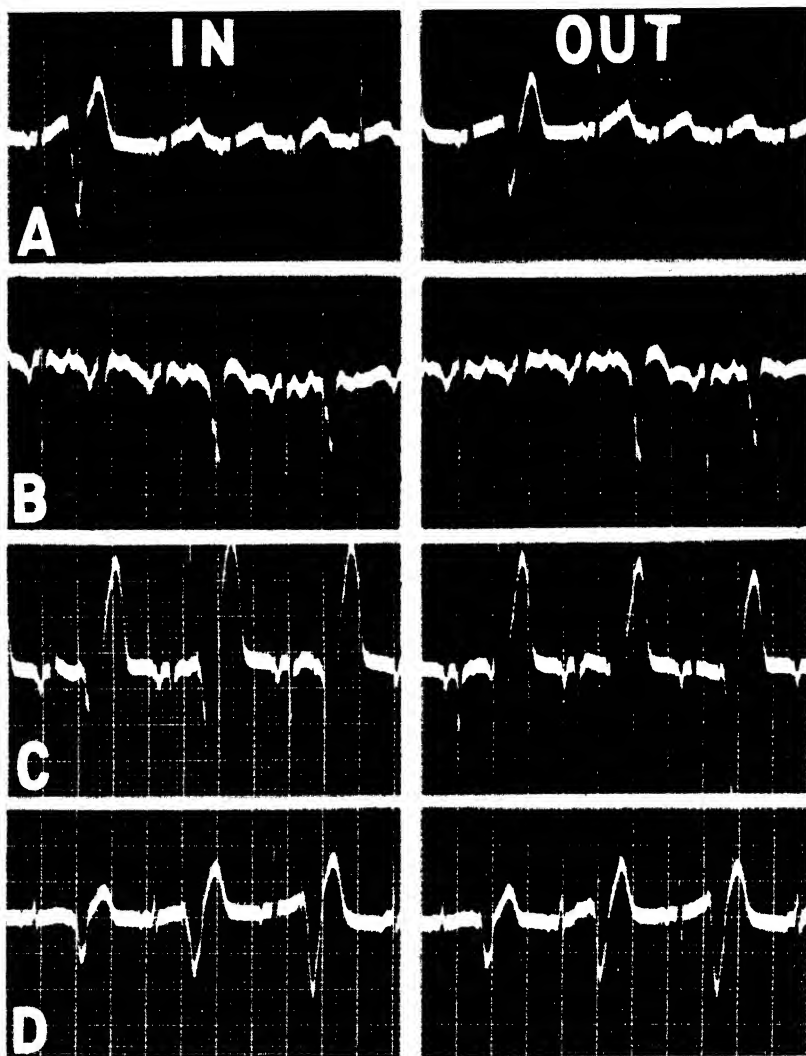


Fig. 1. March 25, 1945. Dog, 18 kgm. Electrodes on the lateral margin of the right ventricle, 1 cm. from the base. A. 4R, B. 4F, C. transthoracic, D. CV. In all cases the chest electrode was immediately external to the stimulating electrodes and in C. the stimulating electrodes were lined up with the transthoracic recording electrodes. In each case the extrasystoles start with a simple QS complex regardless of whether they arise in the endocardium or the epicardium.

can occasionally, but not invariably, be observed when the stimulating electrode is considerably out of line with the recording electrodes in the transthoracic lead or at a distance from the chest electrode in the other leads studied. These

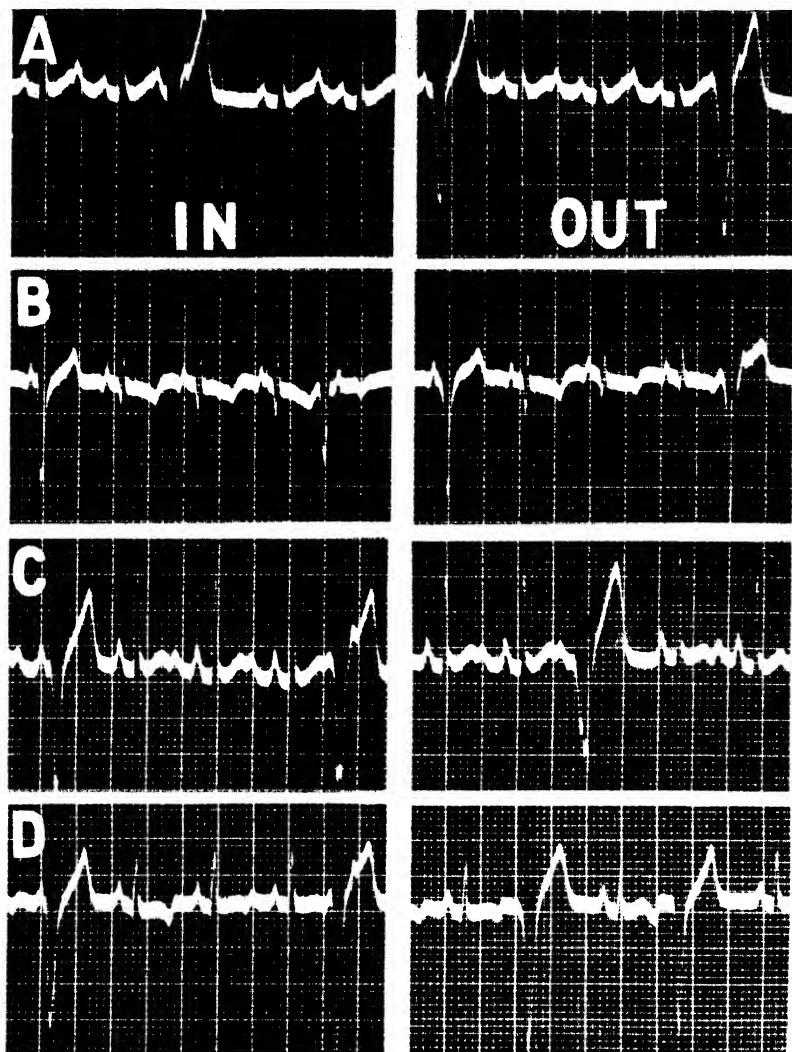


Fig. 2. March 23, 1945. Dog, 10.9 kgm. As in figure 1, except that the stimulating electrodes were applied to the lateral margin of the left ventricle near the apex, and the chest electrode was directly external. A. 4R, B. 4F, C. transthoracic, D. CV. In this experiment also the initial complex is always a simple QS complex in both endocardial and epicardial extrasystoles.

are conditions in which an outwardly travelling electrical potential would be the least likely to be recorded. Further evidence against this explanation of the differences in configuration between endocardial and epicardial extrasystoles is also found in the present experiments. In the first place, the additional, small

initial wave which Lewis interpreted as the sign of outwardly travelling electrical dipoles was as often encountered in the epicardial extrasystole when stimulating and recording electrodes were out of line, as in endocardial extrasystoles. Again, minor movements of stimulating or recording electrodes produced such waves in both endocardial and epicardial extrasystoles. In addition, it was often noted in epicardial as well as endocardial extrasystoles, that the configuration of the initial complex in question varied significantly depending upon the phase of the

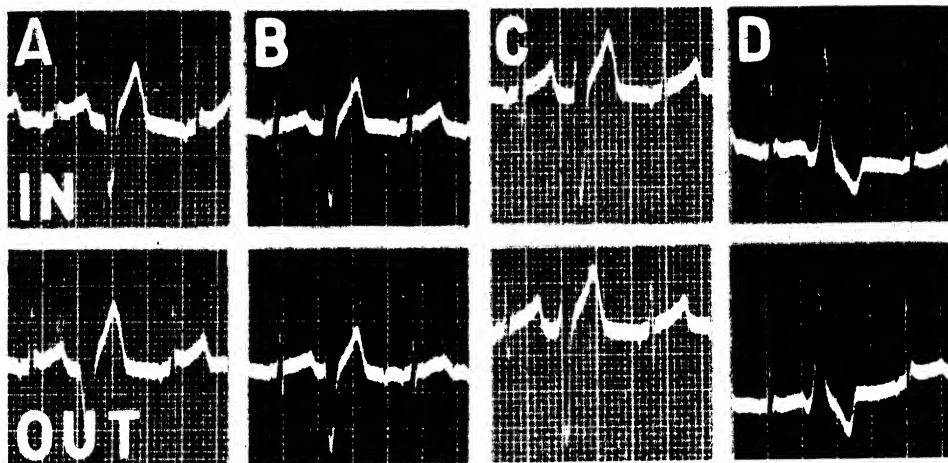


Fig. 3. April 25, 1945. Dog, 11.6 kgm. CV lead with chest electrode immediately external to the right apex.

A. Endocardial extrasystole (above) and epicardial extrasystole (below) from the right ventricle immediately beneath the chest electrode. A simple QS complex appears in both epicardial and endocardial extrasystoles.

B. Endocardial and epicardial extrasystoles from a point midway between A and C. Here the endocardial extrasystole shows an R wave which is hardly visible in its epicardial counterpart. The conclusion of this paper is that the R seen in the endocardial extrasystole here is not the sign of an outwardly travelling wave at the point of stimulation, but simply the first appearance of the R wave that is seen also in C and grows progressively to the height seen in D. It represents the contribution of "extrinsic" factors, i.e., those developing in regions distal to the recording electrode.

C. Endocardial and epicardial extrasystoles from the base of the right ventricle both showing transitional stages in which RS appears.

D. Endocardial and epicardial extrasystoles from the lateral margin of the left ventricle midway between apex and base. Both complexes show a simple R wave.

cycle in which the extrasystoles fell. Early extrasystoles showed the simple R or QS patterns characteristic, according to Lewis, of "epicardial" extrasystoles, while complexes occurring later in the cycle showed variable-sized initial Q or R waves resembling Lewis' "endocardial" extrasystoles. It is evident that each extrasystole started at the same place and was conducted to other regions of the heart from that place. Localized waves of conduction must have been present in all cases, and if the small initial waves seen by Lewis and found in special

circumstances in these experiments represent those locally conducting waves, they should invariably be present. Finally, if the outwardly travelling impulse is reflected in an R wave, this component should be more easily detected in left ventricular extrasystoles of endocardial origin than in those originating in the right ventricle because of the longer conduction pathway in the thicker ven-

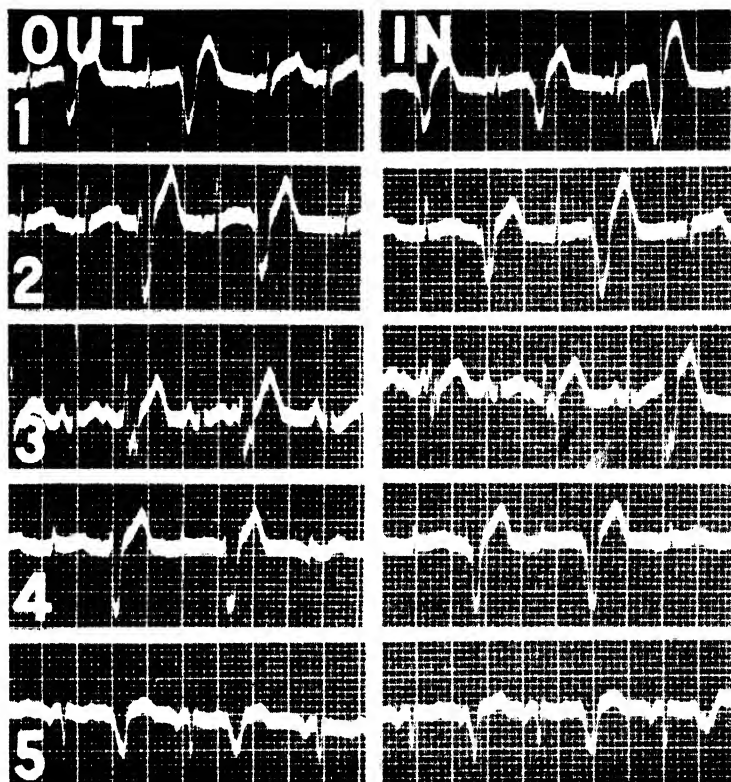


Fig. 4. March 25, 1945. Stimulating electrodes at the right lateral border of the heart midway between apex and base. Records taken with C-V leads with the chest electrode in the following positions: 1, on the right side of the chest immediately above the stimulating electrodes; 2, anterior to 1 and close to the sternum at the same level as 1; 3 caudal to 1 at the lower costal margin; 4 dorsal to 1 and, 5, cephalad to 1, at the level of the first rib. Note that in 2 and 4 an R wave appears in the epicardial extrasystole only.

tricular wall. Actually, it was impossible to detect any differences even in the left ventricle.

A reasonable conclusion to be drawn from these experiments is that the initial waves that can at times be found to distinguish between epicardial and endocardial extrasystoles arise when relatively large segments of the heart at some distance from the recording electrodes become active, i.e., that they represent

an "extrinsic" factor. This explanation has already been shown to account for the occasionally observed differences between endocardial and epicardial extrasystoles as recorded from leads I, II and III (6) and it is supported by the present experiments. Movement of the stimulating electrode away from the chest electrode would augment the contribution to the electrocardiogram of regions distant from the exploring electrode; movement of the chest electrode away from the stimulating electrode would accomplish the same end. Naturally differences between endocardial and epicardial extrasystoles could not occur when the stimulating electrode is as close as possible to the recording electrode, for then the region under the recording electrode would always be the first to be activated. Only when the stimulated area is at a distance, is it possible for the region under the recording electrode to be preceded in activation by another region of the heart.

The variability in configuration related to the location of the extrasystole in the cardiac cycle can be accounted for by assuming that early in the cycle conduction



Fig. 5. March 25, 1945. Stimulating electrodes at the right lateral border of the heart midway between apex and base. Recording electrode (CV lead) on right chest over the right apex (not therefore directly over stimulating electrodes). Epicardial extrasystoles show less and less R the earlier they fall in the cycle.

away from the site of stimulation is slower and that therefore excitation of distant regions occurs relatively later, and these regions do not therefore contribute an early deflection to the electrocardiogram. During the latter part of the cycle recovery is more complete, conduction to distant regions is more rapid and the contribution of such regions may therefore be revealed.

A final indication of the nature of the R waves seen in these experiments is afforded by the progressive transition noticed when the stimulating electrode is moved away from the recording chest electrode or vice versa (fig. 3). In either case the R wave appears in both endocardial and epicardial extrasystoles, and grows at the expense of S, which finally disappears completely.

It thus becomes possible to formulate an interpretation of the several components of precordial electrocardiogram. Excitation of the area underneath the electrode is recorded by a downward movement in the electrocardiogram, representing negativity at the chest electrode. This occurs without regard to the direction of the excitation wave responsible for the activity of the heart muscle under the electrode. This is the only process which can be responsible for down-

ward movement during the QRS component. Thorough exploration of all parts of the epicardium of the entire heart, all of the endocardium of the right ventricle excepting the septum and much of the endocardium of the left ventricle failed to reveal any other regions from which a Q wave could be elicited. These experiments do not, therefore, support Wilson's view that a Q wave results when the "subendocardial muscle of some part of the ventricular wall passes into the active state earlier than the subendocardial muscle which lies between the exploring electrode and the ventricular cavity".

The region of the heart which on stimulation gave complexes showing uncomplicated QS waves was a relatively small area and in all other regions R waves of varying amplitudes were found. They precede an S wave in the region surrounding the area proximal to the chest electrode, but in more distal regions no S was found. The R wave, therefore, represents excitation of distal regions with respect to the chest electrode, which results in a relative positivity of the chest electrode. These distal regions give rise to an R wave whether they are excited from endocardium or epicardium, and it is only by excitation of these distal regions that an R wave can be produced. The claim of Wilson that the R deflection in the "unipolar precordial electrocardiogram" represents the electrical forces produced by excitation of muscle between the chest lead and the ventricular cavity cannot, therefore, be substantiated by these experiments.

SUMMARY

1. Epicardial extrasystoles and their endocardial counterparts have been elicited from the right and left ventricles in the dog and their configuration compared in transthoracic leads, leads 4R and 4F, and in Wilson's central terminal lead.

2. When the stimulating electrodes were carefully aligned with the transthoracic leads or were immediately subjacent to the exploring electrode in the chest leads, no differences were found between endocardial and epicardial extrasystoles, the initial deflections of both showing a simple QS complex.

3. When the stimulating electrodes were not in line with the transthoracic leads or were not directly beneath the exploring electrode in the chest leads, endocardial and epicardial extrasystoles showed one or another of the following configurations:

- a. The same configuration as in 2.
- b. Both epicardial and endocardial extrasystoles showed R waves preceding S.
- c. The endocardial extrasystole alone showed an RS configuration, while the epicardial extrasystole showed a simple QS wave.
- d. The epicardial extrasystole showed an RS, while the endocardial extrasystole showed a simple QS wave.
- e. Extrasystoles (endocardial, epicardial or both) which arose early in the cycle showed a simple QS, while later extrasystoles showed an RS.

4. These results are interpreted to mean that the electrocardiogram, as recorded by limb and chest leads, does not reveal the existence of currents associated with the conduction of excitation from the endocardium to the epicardium.

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SOME FACTORS INFLUENCING THE BIOCHEMICAL APPRAISAL OF GROUP NUTRITIONAL STATUS

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Johnson and co-workers have presented data indicating in part that the fasting rate of excretion of vitamins (ascorbic acid, thiamine, riboflavin, N-methylnicotinamide) in young adults was found to be a more reliable indication of previous intake of those vitamins than was the load test. Therefore it was recommended that in the conduct of nutritional surveys, the fasting urinary levels of vitamins might well be chosen in preference to the load test.

In the course of carrying out several tests of rations used in World War II, we have collected a considerable number of data relating to the dietary intake of nutrients on one hand, and fasting urinary excretion and load test response on the other. These data show that a high correlation occurs also in certain instances between the immediately preceding dietary intake and fasting urinary excretion. Emphasis is placed here on the correlation with *dietary* intake, inasmuch as the data presented by Johnson and co-workers related to the differences observed between the effects of placebo and high synthetic vitamin supplementation to groups of individuals.

In observing this correlation, however, we have noted that for a group of normal young individuals neither showing symptoms of gross nutritional pathology nor receiving any vitamin supplementation, a series of fasting urinary excretions does not always follow the trend of load tests on the same group. Indeed, we have at hand a considerable number of data which could well be interpreted as indicating entirely different states of nutrition, depending on whether the fasting urinary excretion were considered or the load test response. It is the purpose of this communication to present those data which demonstrate the following points therefore:

1. The lack of correlation which may occur between fasting urinary excretion and load test response.
2. Relation of fasting urinary excretion to the *immediately preceding dietary intake*.
3. Possible relation of fasting urinary excretion to other factors, particularly caloric expenditure and environmental temperature.

METHODS. The data referred to specifically were obtained during a ration test lasting approximately eighty days and carried out in a Southern Army camp during April to July 1943. The mean weekly (6:00 a.m. to 6:00 p.m.) temperature ranged from 54°F. at the beginning to a maximum of 86°F. at the end, with

relative humidity varying from an early 43 per cent to approximately 80 per cent beginning in June. The subjects were chosen to represent a typical cross-section of men in the Army, criteria used in attaining this end being medical, sociological, geographical, educational, and occupational (civilian). The test group consisted of 64 officers and men ranging in age from 18 to 42 years, in weight from 128 to 236 pounds, in height from 65 to 74 inches. All were in excellent health as judged by the medical officer in attendance throughout the test, and with few exceptions, all had just previously completed the prescribed 13 weeks of basic military training.

The level of activity for the majority of the command throughout the test was similar in type to that required for infantry basic training. The average caloric expenditure was judged (Pollack, French and Berryman, 1944) to be approximately 3900 calories per man per day. The average daily intake of nutrients for the entire test period was as follows: Calories: 3465, protein: 126 grams, fat: 124 grams, CHO: 461 grams, calcium: 841 mgm., phosphorus: 2025 mgm., iron: 21.7 mgm., vitamin A: 12,000 I.U., thiamine: 1.33 mgm., riboflavin: 1.65 mgm., niacin: 23.1 mgm., ascorbic acid: 98 mgm. Actual analytical values are reported for the vitamins, protein and fat; mineral values were computed from Tables of Food Composition. The average intakes of nutrients varied only slightly between each 10-day period, since the quantity of the ration under test was fixed and distributed equally, and the menus rotated over a 10-day cycle. In the cases of thiamine and riboflavin, the average intakes were less than the men had been receiving before the test was begun.

Biochemical data collected included fasting urinary excretion and load test response for thiamine, riboflavin, and ascorbic acid. The detailed procedures are described below in sequence. Creatinine determinations were carried out routinely on all urine samples as a means of detecting any gross discrepancies in completely voiding the urine.

Load test response. For the ascorbic acid load test, a sterile solution containing 200 mgm. of crystalline ascorbic acid confirmed by analysis was injected intravenously by the attending medical officer six hours following the supper meal, from which all foods high in ascorbic acid had been removed. The load test was begun immediately following the emptying of the bladder, and after the administration of the test dose. The test subjects then resumed their rest, and were awakened six hours later, at which time they voided completely into wide-necked bottles containing metaphosphoric acid preservative. The urine and blood samples were analyzed immediately for ascorbic acid. Three load tests were carried out on days 9, 56 and 77, respectively.

In the case of thiamine and riboflavin, a sterile solution containing 1 mgm. of thiamine hydrochloride, 1 mgm. of the sodium salt of riboflavin (buffered in a borate mixture) and 20 mgm. of nicotinamide was given intramuscularly eight hours following the previous meal. The test subjects had emptied their bladders immediately before. They then resumed their rest, and were awakened four hours later, at which time they voided completely into wide-necked bottles containing acetic acid preservative. The urine samples were immediately stored

in the laboratory ice box and thereafter analyzed as rapidly as possible. Three such load tests were carried out on days 3, 60, and 80, respectively.

Fasting urine excretion. Four-hour fasting urines for the analysis of thiamine and riboflavin in one sample, and ascorbic acid in a separate sample were collected twenty-four hours before load tests, and in addition at intervals of about ten days throughout the eighty-day test. The collections were begun eight hours following the evening meal, and covered the four-hour period from 2:00 a.m. to 6:00 a.m. These urines were also collected in wide-mouth bottles containing the proper preservatives, and were analyzed immediately in the case of ascorbic acid, and as rapidly as possible for thiamine and riboflavin.

Chemical methods:

Urine

Thiamine: Thiochrome method as outlined by Research Corporation Committee on thiochrome. (Hennessy and Cerecedo, 1939)

Riboflavin: Determination of riboflavin by the fluorometric method. (Najjar, 1941)

Ascorbic acid: Determination of ascorbic acid in urine and blood plasma by the use of 2-6 dichlorophenol indophenol. (Evelyn, Malloy and Rosen, 1938)

Creatinine: Determination of creatinine in urine by the colorimetric method of Folin.

RESULTS. A. *Trends of fasting urinary excretion and load test response.* If graphs are drawn representing the group fasting excretions of thiamine, riboflavin and ascorbic acid (see figs. 1, 2 and 3) it becomes quite apparent that there is no consistent trend in any of the instances shown. If single average fasting excretions were considered alone, the widely fluctuating levels would indicate in each instance different degrees of nutritional state in this cross-section of normal young men. This absence of consistent trend is even more striking if the graphs representing load test response are taken into account (see fig. 1, 2 and 3 also). Examination of the latter indicates a definite and progressive overall drop in the load test response in the cases of thiamine and riboflavin, with a slight eventual leveling off in the case of the former. The ascorbic acid load test response remained virtually unchanged until the last twenty days of the test, when it too dropped. It is important to note that the fasting urinary excretion determined just preceding the second load test was in each instance in the higher levels excreted at any time during the test, while conversely, the load test response was considerably lower than it had been originally in the cases of thiamine and riboflavin, and relatively unchanged in the case of ascorbic acid. Thus, if at that time reliance had been placed on the impression gained from fasting urinary excretion alone, it would have appeared that an increase in tissue stores had occurred. That such probably was not the case is shown by the drop in fasting urinary excretion on the next occasion that it was determined, as well as by reference to the load test response and dietary intake levels. When tested statistically, the drops in load test response are found to be highly significant;

conversely, there is no statistically significant trend that can be calculated for the fasting urinary excretions.

There was no correlation between group average fasting urinary excretion and load test response. In the case of the results for each individual in the group also, the coefficient of correlation between any load test response and the immediately

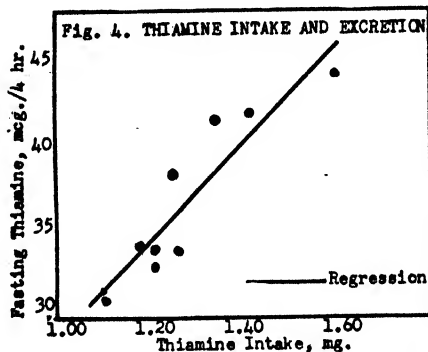
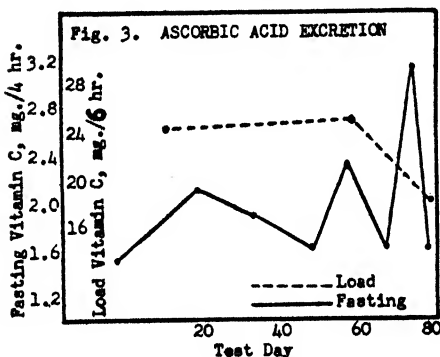
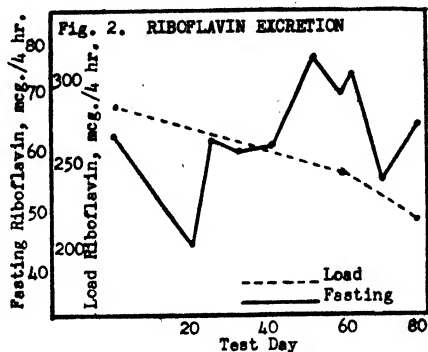
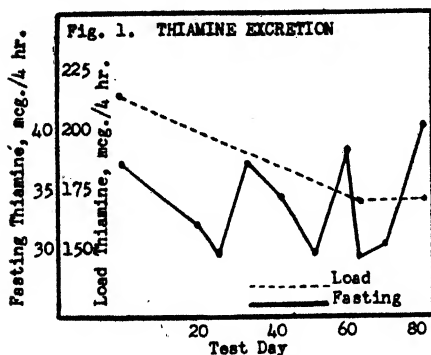


Fig. 1. Group average 4 hour fasting thiamine excretion and 4 hour excretion following intramuscular administration of 1 mgm. thiamine chloride.

Fig. 2. Group average 4 hour fasting riboflavin excretion and 4 hour excretion following intramuscular administration of 1 mgm. riboflavin.

Fig. 3. Group average 4 hour fasting ascorbic acid excretion and 6 hour excretion of ascorbic acid following intravenous administration of 200 mgm. ascorbic acid.

Fig. 4. Group average 4 hour fasting thiamine excretion plotted against group average thiamine intake of the preceding day. Adjustments were made for differences in exercise of the previous day and thiamine intake of the second day preceding.

preceding fasting urinary excretion was quite low (thiamine $r = 0.35$; riboflavin $r = 0.20$; ascorbic acid $r = 0.37$). Although the correlations were statistically significant in the case of thiamine and ascorbic acid, it is obvious that they were not at all decisive.

In brief, whereas load test response might be interpreted to indicate a gradual depletion in the "tissue stores" of thiamine and riboflavin (and perhaps ascorbic

acid in the last twenty days), the fasting urinary excretion of each vitamin showed sudden increases on several occasions. The interpretation of biochemical nutritional state by reference to any single determination of fasting excretion might, therefore, have been quite in error, as judged either by preceding or subsequent fasting excretions throughout the test, and also as interpreted from the load test trend. In addition, comparison of the initial fasting excretion level with fasting levels determined towards the end of the test would indicate a general increase in tissue stores, while load test response would indicate just the opposite. In this regard, it might be necessary to conclude that the two excretions may have been measures or reflections of different things, physiologically speaking. For this possibility we wish to mention the importance of the renal threshold in influencing the levels of load test excretion. It is also possible that the two excretions are influenced by different internal and external circumstances, both physiological and environmental. The possible influence of the latter on fasting urinary excretion will be considered in B and C to follow.

B. *Relation of fasting excretion to immediately preceding dietary intake.* In considering the possible factors that might have been responsible in part for the fluctuations observed in the fasting excretion levels, we have first considered the factor usually assumed to have a direct relationship, namely, the immediately preceding dietary intake. As was indicated earlier, the nutrient intake could not vary markedly on the average between ten-day periods, for the simple reason that similar quantities of the same brands of foods were served beginning with each 10-day menu cycle (i.e., the same foods, cooked in approximately the same manner, were served on days 1, 11, 21, etc., or 2, 12, 22, etc.). However, the fasting urinary excretions were determined at different points within the cycles, and therefore subsequent to somewhat different levels of intake such as may be encountered in any diet pattern.

Generally speaking, fasting urinary excretion was significantly correlated with the immediately preceding dietary intake of the nutrient concerned only in the case of thiamine. Thus the partial correlation of 9 group average fasting urinary thiamine excretions was significantly correlated with dietary thiamine intake on both the day preceding ($r = 0.90$, $P < 0.01$) (see fig. 4), and the second day preceding ($r = 0.86$, $P < 0.05$). We were unable to determine any similar statistical correlation, however, in other instances. Group average basal riboflavin excretion showed some degree of *inverse* correlation with group riboflavin intake of the day preceding ($r = -0.87$, $P < 0.05$). Correlation of group ascorbic acid intake of the day preceding and group urinary ascorbic acid excretion was not significant statistically ($r = 0.54$), but may be adjudged to be indicative of some relationship. It may be mentioned parenthetically that no correlation was found to occur for any of the vitamins between load test response and dietary intake on the preceding or second day preceding.

In brief, strikingly significant correlation was found to occur between fasting urinary excretion and immediately preceding intake only in the case of thiamine. The somewhat questionable significance where riboflavin and ascorbic acid were concerned caused us to consider also the possible effect of other environmental factors. These factors are discussed in C, to follow.

C. *Relation of fasting urinary excretion to other factors.* The possible relationship of fasting urinary excretion to the following circumstances was determined:

1. Individual nutrient intake of the entire diet—protein, fat, carbohydrate and vitamins associated with the one under consideration.
2. Volume of urine in which the vitamins were excreted.
3. Fasting body weight.
4. Surface area of the individual.
5. Estimated daily caloric expenditure.
6. Weather data —6 a.m. to 6 p.m. average temperature and humidity.

Of all of the correlations determined for the factors listed above, only two showed any consistently significant relationship. Exercise (caloric expenditure) showed a high inverse correlation in the case of group fasting thiamine excretion ($r = -0.76$, $P < 0.05$), and there was also some suggestion (not statistically significant, however) of an inverse relationship to mean temperature. In the case of group fasting riboflavin, and exercise, a high direct correlation ($r = 0.90$, $P < 0.01$) was found, and mean temperature also showed a significant correlation ($r = 0.96$, $P < 0.01$).

DISCUSSION AND CONCLUSIONS. In presenting these data and correlations, we have endeavored to point out as a primary purpose that in the appraisal of group biochemical nutritional status, the fasting urinary excretion of vitamins may fluctuate markedly from week to week. Both increased and decreased fasting excretion levels are noted when the diet is relatively constant and while all individuals in the group are apparently undergoing some degree of steady depletion, as judged by load test response. We have investigated by the use of statistical methods, some of the causal relationships that might exist between the fluctuations in fasting urinary excretion, and attendant physiologic and environmental circumstances. We feel that in the case of thiamine, immediately preceding dietary intake may be a chief factor. However, because we have not been able to show that similar relationships did not occur for riboflavin and ascorbic acid, this does not at all mean that they cannot or always do not occur. On the contrary, it is our feeling that there undoubtedly is a high correlation up to a point between the fasting urinary excretion and the preceding dietary intake of a vitamin in the case of extremes of intake, as for example in induced deficiency as described by Keys and co-workers, or where a contrast is made between placebo and synthetic vitamin supplemented groups, as by Johnson et al. On the other hand, our experience indicates that a single determination of fasting urinary excretion should be interpreted with caution in making nutritional surveys of groups or of populations. At the very most, it should be used in conjunction with gross or microscopic clinical appearances, and possibly also with the nutritional appraisal of the diet. Military nutritional survey teams have had considerable success using all three types of data as pieces fitting into a jig-saw pattern.

In presenting data relating to respective correlations between immediately preceding dietary intake, caloric expenditure, and possibly environmental temperature on the one hand with fasting urinary excretion on the other, we wish

to emphasize that they were obtained on a group of young, healthy, and very active adults. The subjects were not transported from an unaccustomed environment to a new one, they were not subjected to sudden vitamin supplementation, and their activity level was not abruptly changed. They were, in short, as closely typical a cross-section of young active adults eating normal foods as could be obtained.

Finally, we feel that the following points in relation to the use of group fasting urinary excretions are worthy of consideration:

1. Some statistical relationship appears to occur in several instances between immediately preceding dietary intake of a nutrient by a group, and the average fasting urinary excretion levels of the same nutrient. This is probably responsible in part for the widely fluctuating levels of fasting vitamin excretion that may be encountered at various times in the same group of individuals. Other factors may also exert an effect, particularly exercise and temperature.

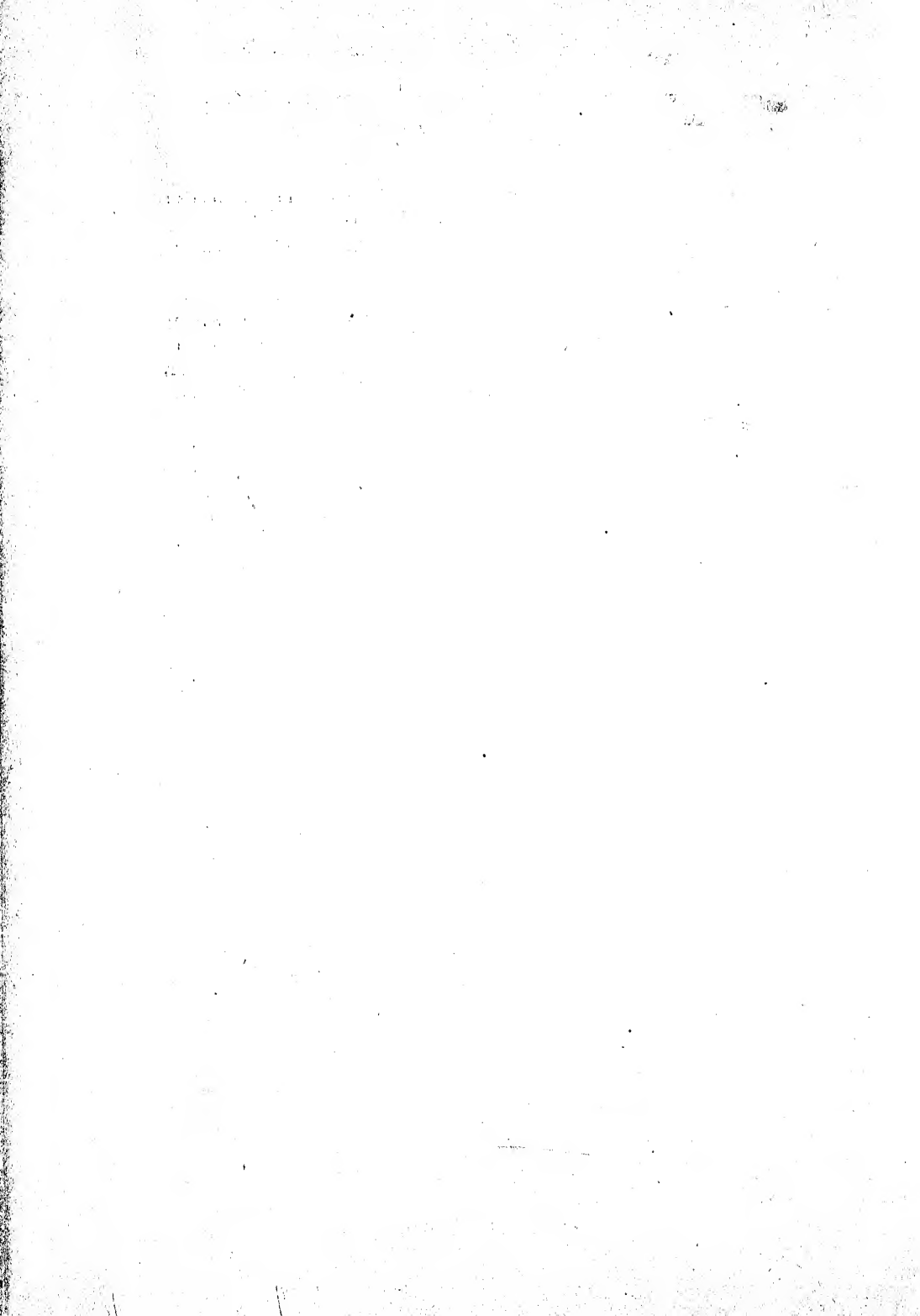
2. In the appraisal of group nutritional status, therefore, it should be kept in mind that an apparently low fasting urinary vitamin excretion could be due entirely to a transient decrease in the intake of that nutrient. Conversely, a high fasting urinary excretion could be due to a transient increase in the intake of that nutrient. Therefore, the use of fasting urinary excretions by reference to arbitrary standards of "normal" and "abnormal" is not alone a safe diagnostic measure in the appraisal of group nutritional state.

3. Certain individuals excrete high, and some low, quantities of nutrients on relatively similar intakes of a nutrient.

4. We have found no statistical correlation between the fasting urinary excretion and the load test response of a group, and but little correlation in the case of individuals.

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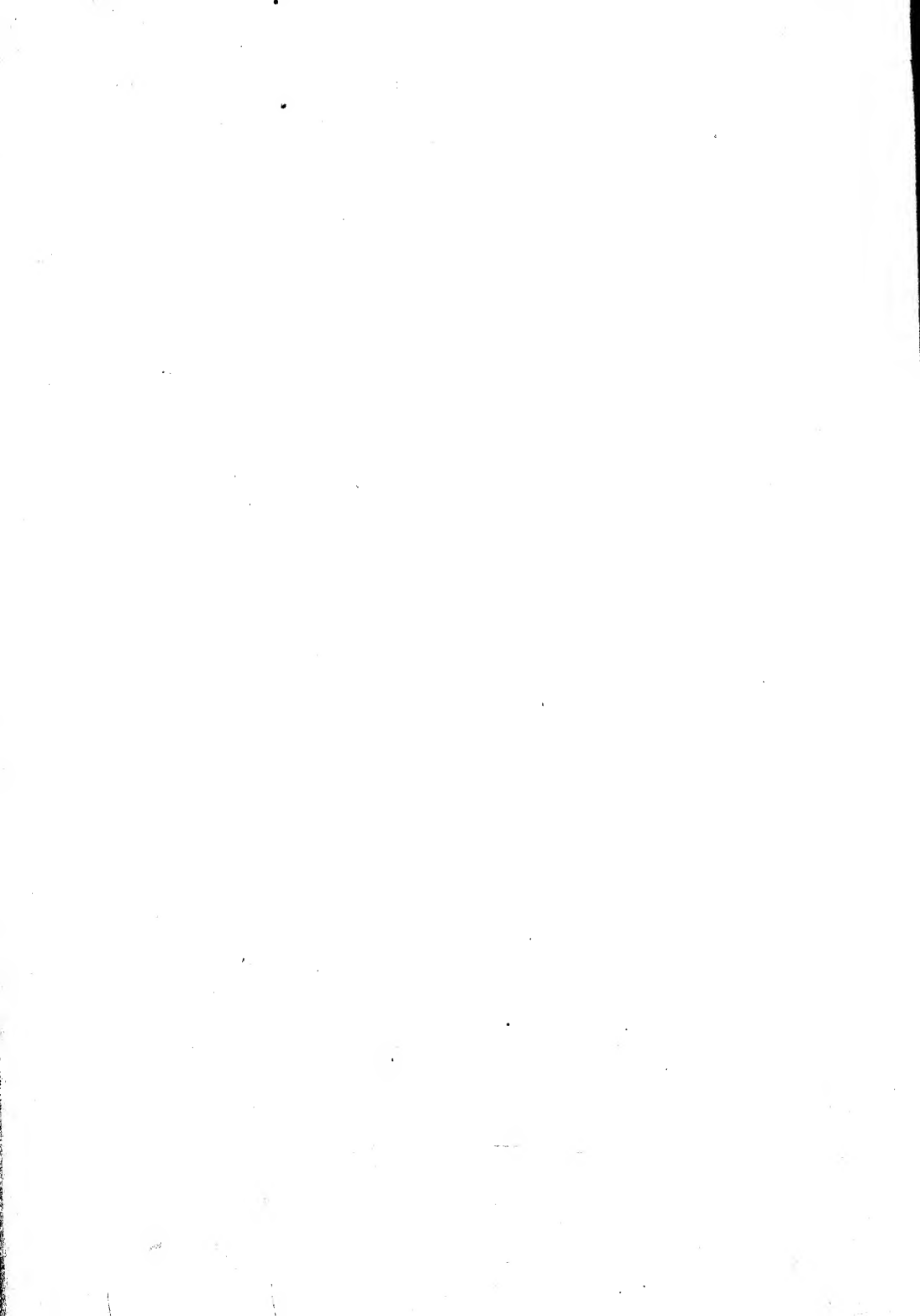
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ERRATUM

Volume 145, page 405: The Effect of Low and High Carbohydrate Meals on the Blood Sugar Level and on Work Performance in Strenuous Exercise of Short Duration, by John Haldi and Winfrey Wynn.

The line (20, p. 405) should read as follows:
was 99, 86, 79 and 85, 83, 85 mgm. per cent respectively.



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THE RENAL CLEARANCE OF PANTOTHENIC ACID

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The urinary excretion of pantothenic acid following its administration to experimental animals has been studied by several investigators (1-3). The published information has not been of sufficient scope, however, to permit conclusions regarding the manner in which pantothenic acid is handled by the renal glomerulus and tubule. During a survey in these laboratories of the renal clearance of a number of compounds, it was observed that the clearance of pantothenic acid was rather unusual. The purpose of this report is to present the relationship between the plasma concentration of pantothenic acid and its renal clearance.

METHODS. The pantothenic acid content of plasma and urine was determined by the microbiological method of Skeggs and Wright (4). The recoveries of pantothenic acid added to plasma and urine were quantitative, within the 5 to 10 per cent error inherent in the method.

Simultaneous creatinine and pantothenic acid renal clearances were determined in dogs in a postabsorptive state using 10 minute urine collection periods. Usually 3 grams of creatinine in solution were administered subcutaneously one-half hour before the clearances were begun. Supplementary doses of 1 gram were given orally at about ninety minute intervals in order to maintain an adequate plasma creatinine concentration. Approximately 500 cc. of water were administered to the dogs by stomach tube with the initial dose of creatinine. At the beginning of the actual experiment, two clearance periods usually were performed before any pantothenic acid was administered. The results of these clearances were considered to represent normal or control values. Thereafter the experiments varied considerably depending on their purpose. In general the purpose was to raise the plasma concentration in predetermined increments over a very wide range in order to determine the relationship of pantothenic acid plasma concentration to its renal clearance. Three or four such increments with two clearances at each level made up the experiment which took from 4.5 to 5.5 hours to carry out. Table 1 has been included for the purpose of presenting a detailed protocol and the results of a representative experiment.

RESULTS. At the prevailing or control plasma concentrations of pantothenic acid, which in these studies were about 0.10 to 0.32 γ /cc., only a minute amount of the vitamin appeared in the urine, as is evident on inspection of either table 1 or figure 1.

If the plasma levels of pantothenic acid are raised carefully and in small increments, an abrupt increase in clearance occurs at a plasma concentration of between 0.5 and 0.8 γ /cc. Once the concentration required to effect saturation

TABLE 1

Protocol: The determination of the relation of plasma concentration to the renal clearance of pantothenic acid. Dog 84, weight 15.9 kgm.

TIME	VOLUME OF URINE	PLASMA CONC.	U/P RATIO	PANTOTHENIC ACID CLEARANCE (UV/P)	GLOMERULAR FILTRATION	PANTOTHENIC ACID FILTERED AT GLOMERULUS	UV _{PA} /GF _{PA} *
hr.:min.	cc./min.	γ /cc.		cc./min.	cc./min.	γ /min.	
0:00	3.0 grams creatinine + 500 cc. H ₂ O, p.o.						
0:50-1:00	2.30	0.32	0.078	0.18	66.0	21.1	0.003
1:00-1:10	2.45	0.23	0.10	0.24	59.7	13.7	0.004
1:15	3.0 mgm. calcium pantothenate/kgm. + 1 gram creatinine + 300 cc. H ₂ O p.o.						
2:10-2:20	3.15	0.67	0.75	2.4	59.4	39.8	0.04
2:20-2:30	3.10	0.70	1.59	4.9	54.9	38.4	0.09
2:35	4.5 mgm. calcium pantothenate/kgm. + 1 gram creatinine + 300 cc. H ₂ O p.o.						
3:30-3:40	2.95	2.6	18.6	54.8	58.6	152.4	1.07
3:40-3:50	2.70	2.8	21.2	57.2	57.1	159.9	1.00
4:00	Priming dose calcium pantothenate 0.25 mgm./kgm., i.v. + 1 gram creatinine, s. c. Constant infusion of calcium pantothenate at a rate of 1 mgm./kgm./min. in 3 cc. saline/min. for the duration of the experiment						
5:10-5:20	3.80	111.0	19.2	72.0	60.3	669.3	1.19
5:20-5:30	4.10	114.0	17.7	72.5	61.4	699.6	1.18

* UV_{PA} = amount of pantothenic acid excreted per minute.

GF_{PA} = amount of pantothenic acid filtered at the glomerulus per minute.

of a significant number of tubules has been exceeded, the renal clearance of pantothenic acid rises abruptly so that at plasma concentrations of about 1.2 γ /cc. the clearance of the compound approximates the glomerular filtration rate (creatinine clearance) of the animal. This is an unusual circumstance. While the clearances of glucose (5) and ascorbic acid (6) approach that of creatinine, in neither instance does the clearance equal that of creatinine. However, at high plasma concentrations the clearance of pantothenic acid equals that of

creatinine, if it be assumed that the clearance ratios slightly greater than 1.0 are due to unavoidable errors exaggerated by the hundred-fold increase in plasma concentration and the commensurate increase in UV values.

At this point it became evident that either the maximal rate of tubular reabsorption of pantothenic acid was much lower than for any agent with which we are familiar, that there must be some peculiarity in the binding of pantothenic acid on plasma proteins, or, that which appeared microbiologically active as pantothenic acid in the blood stream of the resting animal was actually some non-diffusible molecule of which pantothenic acid constituted a part. Several diffusion experiments were set up in order to throw some light on this point.

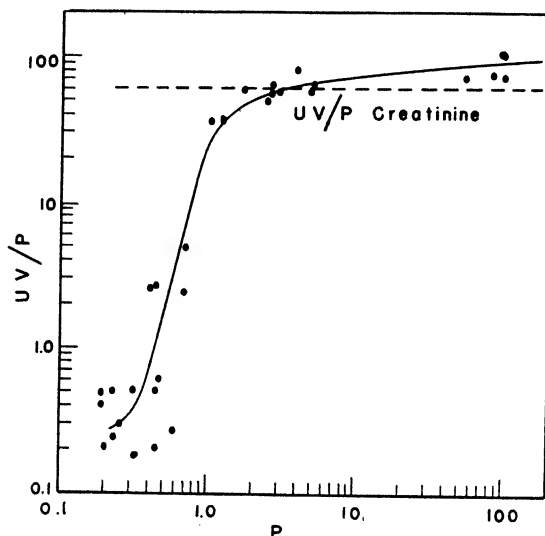


Fig. 1. A curve illustrating the relationship between the plasma concentration of pantothenic acid and its renal clearance. The co-ordinates represent values obtained in seven experiments using two dogs.

In the first of these experiments normal dog plasma was assayed for pantothenic acid both before and after dialysis against 3600 cc. of phosphate buffer at pH 7.4 for 24 hours at room temperature. As indicated in table 2 there was no significant difference between the so-called pantothenic acid content of plasma before and after dialysis, leading one to believe that the pantothenate present was in a nondialyzable state. In a second type of experiment 2 γ /cc. of pantothenic acid were added to a sample of plasma from which an aliquot had previously been removed for assay. This fortified plasma was dialyzed against 3600 cc. of 0.9 per cent saline for 18 hours at room temperature at which time the plasma was reassayed for pantothenic acid. The data substantiated the conclusion that the added pantothenic acid was dialyzed but there remained in the plasma the pantothenic acid-like material which was originally present therein.

A third type of experiment was set up similar to that which has been used for sulfonamide binding studies (7). A known amount of pantothenic acid was added to both plasma and buffer and the system allowed to equilibrate across a Visking membrane over a period of 18 hours at 37°. The results indicate, as illustrated in table 2, that in every instance where pantothenic acid was added in equal concentration to both buffer and plasma there was no indication of the vitamin being bound on plasma protein.

In the light of these experiments on the binding of this agent the results of the renal clearance of pantothenic acid immediately became interpretable. It is

TABLE 2
Diffusion studies of pantothenic acid in dog plasma

EXPERIMENT NUMBER	MATERIAL ASSAYED	PANTOTHENATE CONTENT
		$\gamma/cc.$
1	Plasma	0.31
	Plasma, 25 cc. dialyzed against 3600 cc. of pH 7.4 buffer for 24 hours	0.39
2	Plasma	0.16
	Plasma plus 2 $\gamma/cc.$ of added calcium pantothenate	2.6
	Plasma, 25 cc. plus 2 $\gamma/cc.$ of added calcium pantothenate after dialysis against 3600 cc. 0.9 per cent saline for 18 hours	0.13
3	Plasma	0.16
	Plasma, 8 cc. plus 0.75 $\gamma/cc.$ of added calcium pantothenate after dialysis against	1.02
	Buffer, 16 cc. plus 0.75 $\gamma/cc.$ of added calcium pantothenate for 18 hours.	0.74
	Plasma, 8 cc. plus 2 $\gamma/cc.$ of added calcium pantothenate after dialysis against	2.0
	Buffer, 16 cc. plus 2 $\gamma/cc.$ of added calcium pantothenate for 18 hours.	2.0
	Plasma, 8 cc. plus 100 $\gamma/cc.$ of added calcium pantothenate after dialysis against	107.
	Buffer, 16 cc. plus 100 $\gamma/cc.$ of added calcium pantothenate for 18 hours.	116.

apparent that the predominant amount of pantothenic acid present in normal plasma is in a nondiffusible form and therefore it would be impossible for the material to be filtered and hence be excreted by filtration. When the plasma concentration of pantothenic acid was elevated by the intravenous administration of the agent we were in effect adding a material which appears to be only a precursor of the normally nondiffusible pantothenic acid-like constituent of normal plasma. This added pantothenic acid, being readily diffusible, is filtered at the glomerulus at essentially the same rate as creatinine. Thus, when "free" pantothenic acid was administered the clearances increased rapidly with increasing plasma concentration to equal that for creatinine.

SUMMARY

These data indicate that at ordinary, normal, plasma concentrations only a trace of pantothenic acid is excreted in the urine, hence its apparent renal clearance was of the order of from 0.2 to 0.5 cc./min. Diffusion studies showed that the pantothenic acid present in the plasma of the untreated animal exists in a nondiffusible state. On increasing the plasma concentration of the compound by oral or intravenous administration to about 0.5 γ /cc. there is an abrupt rise in its calculated clearance. When the plasma concentration of pantothenic acid is raised to 1.2 γ /cc., its renal clearance approximates the glomerular filtration rate of the animal. Further elevation of the plasma concentration of pantothenic acid to or above 100 γ /cc. does not alter materially the clearance ratio.

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THE NATURE OF VOLUME EFFECTS IN RENAL CLEARANCES

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The existence of a causal relationship between the rate of urea excretion and the rate of urine flow at reduced volumes has been thoroughly established by an abundant literature stemming from the work of Austin, Stillman and Van Slyke (1). Some evidence of similar conditions affecting the excretion of creatinine was found by Holten and Rehberg (12) and by Chesley (6). More recently, analogous effects have been demonstrated for uric acid by Brøchner-Mortensen (3) and for potassium by Hall and Langley (11). A question arises as to whether dependency between excretion rate and urine flow is a general phenomenon.

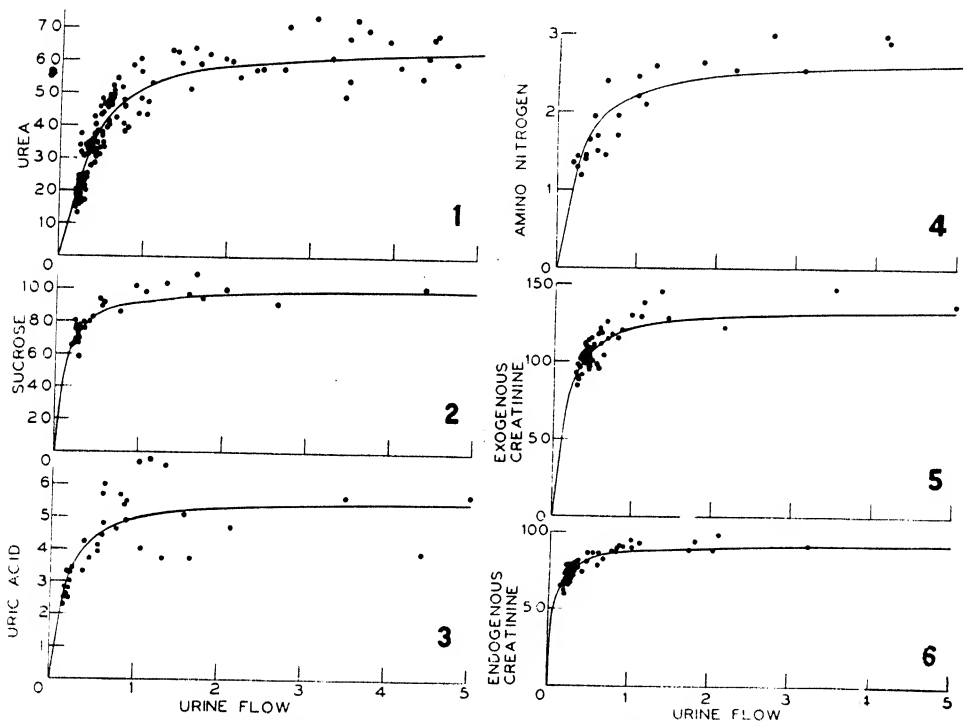
The purpose of this paper is to report a study of the effect of urine flow on the plasma clearances of several substances: urea, sucrose, uric acid, amino-nitrogen, creatinine, inulin, sulfadiazine and sulfathiazole.

EXPERIMENTAL. All experiments were performed on a trained fasting subject (RFH), 30-32 years of age and having a calculated surface area of 2.06 sq. m. Catheterization was avoided in order to eliminate reflex effects upon renal activity. Preliminary dehydration varied from 12 to 30 hours. Urine collection periods ranged from 30 to 40 minutes. Ingestion of tap water was relied upon to obtain any desired elevation of urine flow during the experiments. All calculations relating to sub-maximal clearances were based on data obtained when the rate of urine flow was falling.

Urea determinations were performed gasometrically with the hypobromite reagent of Van Slyke and Kugel (21). Plasma filtrates were prepared with the ferric sulfate barium carbonate reagents of Steiner, Urban and West (20); as modified by White and Monaghan (23). These filtrates were found to require a correction of 2 mgm. per 100 cc. for non-urea nitrogen. Creatinine was determined by the method described by White and Monaghan (23). Inulin was estimated by the method of Corcoran and Page (7), as modified by White, Heinbecker and Rolf (22). Sucrose determinations were made with the reagent used for inulin, by means of a calibration curve obtained with standard solutions of sucrose. Uric acid was determined by the method of Bulger and Johns (4). Plasma amino-nitrogen was estimated by the methods of Folin (10), urine amino-nitrogen by the procedure of Northrop (16). Sulfadiazine and sulfathiazole were determined in the free form by the method of Bratton and Marshall (2). Photoelectric colorimeters were used in all determinations where applicable.

The experimental results are shown graphically in figures 1-9, inclusive. The effect of urine flow on the urea clearance, figure 1, appears similar to that originally described by Austin, Stillman and Van Slyke (1); while the uric acid clearance

data, figure 3, agrees substantially with the observations of Brøchner-Mortensen (3). Analogous volume-clearance effects are shown for the other substances tested. This suggests that such effects may be encountered generally and irrespective of the magnitudes of maximal clearances.



Figs. 1-3. Clearances of urea, sucrose and uric acid in relation to urine flow, in cubic centimeters per minute corrected for surface area. Curves represent theoretical equations fitted to experimental data.

Figs. 4-6. Clearances of amino-nitrogen, exogenous creatinine, and "endogenous creatinine" in relation to urine flow, in cubic centimeters per minute corrected for surface area. Curves represent theoretical equations fitted to experimental data.

Exponential relationship of clearance and urine flow. Dole (8) has shown that volume-clearance effects for urea can be represented accurately by means of an exponential equation based on the laws of diffusion:

$$(1) \quad Cl_u = F \times \phi \times e^{-k_2 \cdot V}$$

where Cl_u is the plasma urea clearance, F is the glomerular filtration rate, ϕ is the fraction of filtered urea which is not reabsorbed in the proximal tubules, k_2 is the product of permeability coefficient and internal surface area of the distal

tubules, and V is the rate of urine flow. This expression can be arrived at in a simple and direct manner by assuming that

$$t = \frac{k}{V}$$

where t is the time required for a segment of urine to traverse a distal tubule and k is a constant of proportion. This type of relationship was suggested by Shannon (18) and would necessarily follow if no water were reabsorbed in the distal tubules. The rate of diffusion loss from distal tubules delivering a substance in concentration u should be

$$-\frac{du}{dt} = k_1 u$$

where k_1 contains the product of diffusion coefficient and available surface, in addition to membrane constants.

Integrating:

$$u = u_0 e^{-k_1 t}$$

Multiplying by V and substituting k/V for t :

$$(2) \quad uV = u_0 V e^{-k_2/V}$$

which is the equation obtained by Dole (8) for urea under conditions where water reabsorption is negligible. It has been found possible to describe satisfactorily the relationship between urine volume and the various renal clearances investigated in this study, by means of analogous exponential equations, shown graphically in figures 1-9, inclusive.

These equations were fitted to the experimental data in two steps: First, the rate of urine flow corresponding to the "augmentation limit" for a particular substance was determined roughly by inspection and the maximal clearance, C_0 , estimated as the mean of clearance values above this limit: 0.5 cc. per minute for inulin; 1 cc. per minute for uric acid, sucrose, creatinine, sulfadiazine, and sulfadiazine, and sulfathiazole; 2 cc. per minute for urea and amino-nitrogen. Second, the exponential constants were evaluated by substituting experimental values for V and C into the general equation:

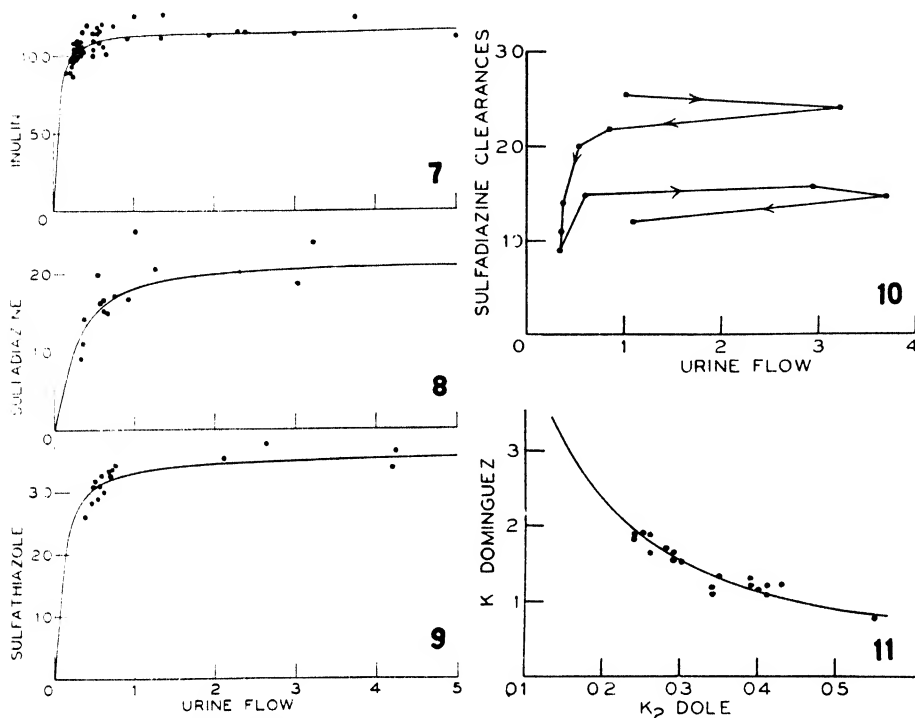
$$(3) \quad k_2 = V (1n C_0 - 1n C)$$

Constants obtained by this method are given in table 1. The constant for urea is of the same order of magnitude as those obtained by Dole (8). Table 2, compiled from the data of Møller, McIntosh and Van Slyke (15), provides a further comparison.

Evidence for diffusion effects in low-volume clearances. As suggested above, it seems likely that the exponential constant k_2 in the equation describing the volume-clearance effect may contain the diffusion coefficient of the substance under consideration. If the other components of k_2 are constant, it should follow that

$$\frac{k_2}{D} = \text{Constant}$$

This relationship has been tested by the substitution of experimental values, as shown in table 1. Examination of the data suggests a simple relationship between the exponential constants and diffusion coefficients in the case of urea,



Figs. 7-9. Clearances of inulin, sulfadiazine, and sulfathiazole in relation to urine flow, in cubic centimeters per minute corrected for surface area. Curves represent theoretical equations fitted to experimental data.

Fig. 10. A typical experiment, showing the relation of sulfadiazine clearances to urine flow, in cubic centimeters per minute corrected for surface area. The plotted points represent clearances estimated during consecutive 30 minute intervals, while the connecting lines and arrows indicate the course of the experiment. The incomplete reversal of clearances with rising urine flow following a drop to low levels is evident. A return to ordinary maximal clearance levels during an experiment of this type requires approximately 24 hours.

Fig. 11. Relation between exponential constants in equations describing volume-clearance effects for urea, as formulated by Dole (8) and Dominguez (9). Plotted points are calculated for individual subjects from the data of Møler, McIntosh and Van Slyke (15). Curve represents approximate regression formula: $kk_2 = 0.45$.

sucrose, uric acid, amino-nitrogen, exogenous creatinine, inulin and sulfathiazole clearances. The clearances of "endogenous creatinine" and sulfadiazine do not conform to this relationship. These exceptions will be considered in some detail.

In the case of "endogenous creatinine," the volume-clearance effect differed so much from that found for exogenous creatinine as to cast some doubt on the identity of these substances. Smith, Finkelstein and Smith (19) have previously questioned the chemical unity of "endogenous creatinine" in the plasma because

TABLE 1

Maximal clearances and exponential constants in theoretical equations describing volume-clearance effects

Mean values are given, with reference to equations of the type:

$$C = C_0 e^{-k_2/V}$$

SUBSTANCE	MAXIMAL CLEARANCE C_0	k_2	DIFFUSION COEFFICIENT* D	k_2/D
	cc./min./1.73 sq. m.		/sq. cm./day	
Urea.....	66	0.25	1.33	0.19
Sucrose.....	100	0.08	0.55	0.15
Uric acid.....	5.6	0.13	0.80	0.16
Amino-nitrogen.....	2.7	0.23	1.19	0.19
Creatinine (exogenous).....	135	0.11	0.85	0.13
Creatinine (endogenous).....	92	0.05	0.85 (?)	0.06
Inulin.....	116	0.035	0.20	0.18
Sulfadiazine.....	22	0.20	0.64	0.31
Sulfathiazole.....	36	0.08	0.64	0.13

* Diffusion coefficients for urea, sucrose, creatinine and inulin are the values obtained by Bunim, Smith and Smith (5); the coefficient for uric acid is that given in the International Critical Tables; the coefficients for amino-nitrogen (as glycine) and the sulfonamides are calculated by Graham's Law from the coefficients for urea and sucrose, respectively.

TABLE 2

Values for urea k_2 calculated from the data of Møller, McIntosh and Van Slyke (16)

SUBJECT	k_2	SUBJECT	k_2
Austin.....	0.40	F. C.....	0.35
Van Slyke '21.....	0.39	E. V.....	0.34
Van Slyke '27.....	0.30	C. A.....	0.25
J. F. M.....	0.41	G. S.....	0.26
L. L.....	0.26	J. S.....	0.43
W. N.....	0.41	H. C.....	0.28
J. C. B.....	0.55	C. D.....	0.39
A. H.....	0.34	J. P.....	0.29
Rehberg.....	0.24	W. G.....	0.29
McLean.....	0.24	Mean.....	0.34

of variations in clearance values with different methods of plasma protein precipitation. The behavior of the "endogenous creatinine" clearance suggested that at least part of the chromogenic substance in the plasma is of higher molecular weight, i.e., less diffusible, than ordinary creatinine.

Urine volume effects on sulfadiazine clearances were found to be greater than expected, and were only slowly reversible, as shown in figure 10. The most likely explanation appeared to be a progressive injury to the renal epithelium with decrease in urine flow, leading to increased back-diffusion. This observation may have some clinical significance.

During the course of this study it was found that exponential equations of the type developed by Dominguez (9) could be fitted rather satisfactorily to the experimental data. The equations of Dole (8) and Dominguez (9) do not appear to be commensurate, although there is a tendency toward an inverse relationship between the exponential constants, shown for urea in figure 11. Correspondingly, an inverse relationship between exponential constants and diffusion coefficient

TABLE 3

Relation between reabsorption rates and diffusion coefficients

Each reabsorption rate is calculated as the difference between the filtration rate (inulin clearance = 120 cc./min.) and the maximal plasma clearance, C_o .

SUBSTANCE	MAXIMAL CLEARANCE* C_o	REABSORPTION RATE R	DIFFUSION COEFFICIENT† D	R/D
	cc./min.	cc./min.	/sq. cm./day	
Urea.....	68	52	1.33	39
Sucrose.....	100	20	0.55	36
Xylose.....	94	26	0.83	31
Creatine.....	94	26	0.79	33
Ferrocyanide.....	68	52	1.5	35

* The urea clearance is from Dole (8); the sucrose clearance is from Keith, Power and Peterson (13); the xylose clearance is the mean of values obtained by Pitts (17) and by Keith, Power and Peterson (13); the creatine clearance is equal to the xylose clearance, according to the data of Pitts (17); and the ferrocyanide clearance is equal to the urea clearance, as observed by Miller and Winkler (14).

† The diffusion coefficients for xylose and creatine are calculated by Graham's Law from the coefficients for sucrose and creatinine (5), respectively. The diffusion coefficient for sodium ferrocyanide is estimated indirectly from the value of 1.07/sq.cm./day at 18° C. given for potassium ferrocyanide in the International Critical Tables, assuming equal diffusibility of the sodium salt and a temperature coefficient of around 0.025 (Øholm's rule).

cients is apparent when equations of the Dominguez type are fitted to the data, although the results are somewhat less consistent than those shown in table 1.

Possible diffusion effects in maximal clearances. It appears that most substances excreted by the kidneys have maximal clearances below the filtration rate, indicating some reabsorption from the glomerular filtrate. The rate of reabsorption, R , of a freely filtrable substance should be measured by the difference between its maximal clearance, C_o , and the filtration rate (inulin clearance), F :

$$R = F - C_o$$

In the case of substances whose reabsorption depends chiefly on back-diffusion alone, it seems probable that a simple relationship might exist between reabsorp-

tion rates and diffusion constants. The data presented in table 3 suggest that reabsorption may be proportional to diffusibility as a factor determining the magnitudes of the maximal clearances of a few substances. Such a state of affairs seems to accord with the concept of two-stage water reabsorption, producing concentration gradients that are constant in the proximal tubules and variable in the distal tubules, with corresponding losses by back-diffusion. It is obvious that the excretion of most substances, including uric acid, amino-acids, glucose, sulfonamides, hexitols, electrolytes, etc., cannot be explained by this simple concept and must in all probability be conditioned by more complex processes in the tubular epithelium.

SUMMARY

1. Analogous effects of reduced rates of urine flow on plasma clearances are demonstrated for urea, sucrose, uric acid, amino-nitrogen, creatinine, inulin, sulfadiazine and sulfathiazole. The similarity of these effects suggests that they represent a general phenomenon.

2. The volume-clearance effect in the case of each substance investigated may be represented satisfactorily by an exponential relationship of the type which Dole (8) developed for urea.

3. In accordance with a general derivation of Dole's equation, it is shown that volume-clearance effects for the substances examined tend to vary with their respective diffusion coefficients. Apparent exceptions are noted for "endogenous creatinine" and sulfadiazine, for which possible explanations are submitted.

4. Evidence is presented which suggests that the variation of the maximal clearances of a few substances from the filtration rate may correspond with differences in diffusibility of these substances.

Grateful acknowledgement is made to Dr. Harvey L. White for his advice and encouragement during the early phases of this study and to Prof. Joseph Erlanger for facilities furnished by the Department of Physiology of the Washington University School of Medicine.

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EFFECT OF PROTEIN DEFICIENCY AND CHOLESTEROL FEEDING ON THE LIVER OF DOGS

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The protein fraction of the diet exercises a controlling influence on the amount of liver lipids. Its lipotropic effect has been clearly shown by various authors (1-11) in studies made on rats. Singel and Eckstein (12) showed the same effect in mice. Elman and Heifetz (13) presented histological evidence of fat infiltration and loss of cytoplasm from the liver cells of dogs kept on a carrot rich protein-deficient diet for six weeks. The livers were analyzed but no individual values for lipids were given. Aside from some work done in this laboratory on dogs by Hough, Monahan, Li and Freeman (14), little information can be found in the literature concerning the fatty changes in the liver due to protein deficiency in dogs.

"Cholesterol" fatty liver has been produced by feeding large amounts of cholesterol to rats (15-23), rabbits (23-27), guinea pigs (23, 28, 29), chickens (30), and cats (31). Cholesterol has been fed to dogs in an attempt to produce lipemia (32, 33), but no fatty liver produced by this means in dogs has been reported to our knowledge.

Hough and Freeman (34) have shown how the serum phosphatase and hepatic dye clearance of dogs are influenced by protein in the diet. Hough, Monahan, Li and Freeman (14) studied further the effect of choline and cystine on the liver function of protein-deficient dogs. MacLean, Ridout and Best (35) found no relation between bromsulfalein excretion and fat content of the liver in rats. McKibbin, Thayer and Stare (36) derived a similar impression from choline-deficient puppies.

The present study was planned with the following aims: to determine *a*, the effect of protein depletion upon liver lipids as well as liver function and serum phosphatase in dogs; *b*, the effects of cholesterol feeding in dogs, and *c*, whether or not there is any relation between serum phosphatase, dye clearance and lipid concentration in the liver.

PROCEDURE. Twenty adult male dogs were fed a high-fat protein-deficient diet which contained lard 33 per cent, sucrose 55 per cent, dried brewers' yeast 5 per cent, ground cellophane 5 per cent, Wesson's salt mixture (37) 2 per cent, and 10 drops of percomorph oil per kilo of food. This diet is practically free from protein except for that contained in yeast. Animals were fed 40 Calories per pound body weight daily except Sundays. In some cases food left was weighed, so that the actual consumption could be calculated.

Ten of these animals received 0.1 gram cholesterol¹ per pound body weight per

¹ Dr. David Kelin, director of the Wilson Laboratories, Chicago, kindly supplied us with the cholesterol used in this study.

day, given in gelatin capsules just before the meal. Three others of this group were given 0.2 gram of cholesterol daily.

As controls, another group of fourteen dogs were fed with an isocaloric diet which supplied 2 grams of protein per pound of body weight per day. In this diet, 25 per cent casein was substituted for the same amount of sucrose in the protein-deficient diet. Seven of the controls received the same amount of cholesterol (0.1 gram per pound) as did ten dogs in the protein-deficient group.

All animals were kept in the laboratory under observation and were fed Pard or dehydrated Pard² for at least two weeks before control data were taken and before they were put on the respective experimental regimens. All the animals were weighed every week before and during the experimental period.

Usually, the hepatic dye clearance and serum phosphatase were determined before and every two weeks during the course of the experiment. Serum phosphatase was determined by Bodansky's method (38). For the dye clearance, the Rose Bengal test of Stove, Delprat and Weeks (39) modified by Hough and Freeman (34) was used. The total fatty acids and the total cholesterol of the serum were determined also and are reported in another communication (39a).

Protein-deficient animals were sacrificed by injecting nembutal when they were moribund, and a careful autopsy was made immediately in every case. The fresh liver was weighed, and total lipids were determined in duplicate by the method of Best, Channon and Ridout (40). The extracted lipids in these samples were dissolved in chloroform, and aliquots were taken to determine the total cholesterol content, according to Hoffman's method for blood (41). Microscopic findings of livers and other organs will be reported elsewhere. Control animals were sacrificed and treated in a similar manner after sixteen weeks, which was near the maximum survival period of the protein-deficient animals.

Two other dogs were on starvation (with water ad libitum) till they were moribund. They were weighed, hepatic dye clearance was determined every week, and serum phosphatase was determined on two occasions. Their livers were also weighed and analyzed for lipids.

In addition to the longer term experiments described above, some short time observations were made to determine how early a diet may influence the function or composition of the liver. The procedures and treatment of these animals were as described above. In addition to the diets mentioned previously, larger amounts of cholesterol and of protein were fed to demonstrate the maximal effect of each substance in relation to the other.

The diets used in the short time experiments are summarized below:

1. High-fat protein-deficient diet.
2. High-fat protein-deficient diet + 0.1 gram cholesterol per pound body weight daily.
3. High-fat protein-deficient diet + 0.5 gram cholesterol per pound body weight daily.
4. High-fat control (25 per cent protein) diet + 0.1 gram cholesterol per pound body weight daily.

² Dog food prepared by Swift & Co., Chicago.

5. High-fat control (25 per cent protein) diet + 0.5 gram cholesterol per pound body weight daily.

6. High-fat 40 per cent protein diet + 0.1 gram cholesterol per pound body weight daily.

The high-fat protein-deficient diet and the high-fat control diet have already been described. The high-fat 40 per cent protein diet was one in which a part of the sugar of the protein-deficient diet was replaced by casein, and which supplied 3.2 grams protein per pound body weight. The cholesterol supplement was either given in gelatin capsule just before the meal or else mixed with the food.

The Rose Bengal clearance and serum phosphatase were determined before the experiment and on the second, fourth and seventh days of the short term experiment. The animals were killed with nembutal the day after the last feeding and were autopsied. Specimens of various organs were preserved for microscopic examination, the findings of which will be reported separately. The livers were weighed and were analyzed for total lipids and total cholesterol.

RESULTS. *Part I—Long Term Experiment.* *Survival period and physical conditions.* The survival period of individual dogs is listed in tables 1A and 1B. The protein-deficient dogs survived 10 to 18 weeks, average 14. Though quite a number of lower figures fell in the group with cholesterol, yet there was no great difference with or without this supplement. The average for the former was $13\frac{1}{2}$ weeks, while for the latter it was $14\frac{1}{2}$ weeks. Two animals on starvation survived for 9 weeks.

The average weight loss of the protein-deficient animals irrespective of whether or not they received cholesterol was about the same. Some dogs were unable to maintain their weight as early as the second or third week and, on the other hand, a few dogs did not lose more than one and a half pounds in weight even up to the twelfth or thirteenth weeks, but most animals began to have significant changes in weight between the fourth and eighth weeks. One dog on starvation lost $14\frac{1}{2}$ pounds and the other one lost $11\frac{1}{2}$ pounds.

Anorexia was a common symptom in all the depleted dogs whether fed cholesterol or not. Most of them began to leave some food between the sixth and tenth weeks. Some were in a state of fasting during the last few days of life.

All dogs on the protein-deficient diet became anemic and appeared dehydrated, and nearly all developed trophic ulcers on their extremities. Some specimens of their urine contained bile-pigments and proteins. Hyperbilirubinemia and edema of the legs were detected in a few of these depleted dogs. Autopsies revealed that nine of these twenty animals had duodenal ulcer. This aspect of the work is reported elsewhere (41a).

Analysis of liver. In tables 1A and 1B are also included weights of the livers, their total lipid and cholesterol content, and the ratio of the two expressed in percentage ($\text{cholesterol} \div \text{total lipids} \times 100$). The relation between the initial and final body weights and the liver weights are graphically represented in figure 1, while in figure 2 is shown the relation between the total amount of liver lipids and of liver cholesterol and the final body weight.

Size of liver. The weight of the livers of the dogs on a high-fat diet with 2

grams casein per pound body weight varied from 8.4 grams to 14.8 grams, an average of 10.9 grams per pound final body weight. Cholesterol given with 2 grams per pound of protein in the diet had no significant effect on the size of the liver. The livers of the protein-deficient animals (without cholesterol) were enlarged when expressed in relation to their final body weight. Protein deficiency

TABLE 1A

DOGS	SURVIVAL PERIOD	MINIMUM DYE CLEARANCE	MAXIMUM P-TASE	LIVER					
				Weight	Total lipids		Cholesterol		'sterol lipids
					Conc.	Per lb.†	Conc.	Per lb.†	
High-fat protein-deficient diet									
	<i>weeks*</i>	<i>per cent</i>	<i>units†</i>	<i>gram</i>	<i>per cent</i>	<i>gram</i>	<i>per cent</i>	<i>milligram</i>	<i>per cent</i>
C1	14.5	50	39.5	325	12.0	1.81	0.24	36	2.0
C19	14	42		224	25.8	4.20	0.38	62	1.5
C20	12	36		330	31.9	6.00	0.24	45	0.7
C21	17.2	52		198	7.9	1.04	0.25	33	3.2
C22	16	37		205	14.9	1.53	0.28	29	1.9
C31	16	61	10.5	209	6.6	0.76	0.29	30	4.3
C32	12	1	83.7	372	14.4	2.06	0.23	33	1.6
High-fat protein-deficient diet with cholesterol									
C2.	14.3	54	22.6	392	28.7	6.85	3.50	836	12.2
C3	13	35	26.2	312	22.3	6.05	1.29	350	5.8
C4	13	55	20.2	255	22.4	4.57	0.94	192	4.2
C5	12.4	58	30.8	183	17.7	2.94	0.59	98	3.3
C10	12.5	43	13.3	299	20.5	3.00	1.24	181	6.1
C11	11.3	47	20.8	373	32.5	7.55	2.16	503	6.6
C12	14.5	41	16.6	602	43.0	14.79	2.46	846	5.7
C26	10	36		533	35.5	9.49	2.73	729	8.0
C27	16	61		407	28.9	6.18	1.25	268	4.3
IT	18	41	4.3	150	11.6	1.39			
High-fat protein-deficient diet with double dose of cholesterol									
C14	15	37	59.9	286	19.8	4.93	1.09	271	5.5
C42	16	44	38.1	557	35.5	9.10	1.37	351	3.9
C43	9.4	47	50.1	530	16.7	3.51	4.09	856	24.5

* Figures after decimal points indicate number of days.

† Bodansky units per 100 cc. serum.

‡ Per lb. body weight.

with cholesterol caused a still more marked enlargement of the liver. This was clearly shown by the total weight as well as when expressed in terms of percentage of body weight. Starvation did not cause the liver to shrink to the same extent as the body as a whole. The livers of these two animals were much smaller than any of the others yet were relatively larger than the livers from protein-deficient dogs without added cholesterol when calculated on a body weight basis.

Total liver lipids. The liver lipid content of the dogs on the control diet varied from 5.2 per cent to 7.4 per cent with an average of 6.2 per cent. Two grams of casein per pound body weight daily did not prevent fatty infiltration in all animals when cholesterol was added to the high-fat diet. The livers from three of these animals had no more fat than those on the same high-fat control diet without this supplement; two of them contained slightly more lipids than normal

TABLE 1B

DOGS	SURVIVAL PERIOD	MINIMUM DYE CLEARANCE	MAXIMUM P-TASE	LIVER					
				Weight	Total lipids		Cholesterol		'sterol lipids
					Conc.	Per lb.†	Conc.	Per lb.†	
High-fat control diet (2.0 grams protein per lb. body weight daily)									
	weeks*	per cent	units†	gram	per cent	gram	per cent	milligram	per cent
C16	13‡	108	1.8	385	5.2	0.77	0.29	43	5.6
C28	16	83		238	5.7	0.59	0.26	27	4.5
C29	17.4	90		224	6.0	0.61	0.27	28	4.5
C30	16	102		258	6.1	0.59	0.26	26	4.2
C37	16	97	1.7	307	7.4	0.62	0.34	29	4.6
C38	16	94	2.3	406	5.7	0.66	0.31	36	5.5
11E	31	94	8.1**	329	7.3	0.82	0.28	28	3.8
High-fat control diet (2.0 grams protein per lb. body weight daily) with cholesterol									
C6	19‡	91	2.6	360	6.8	0.86	0.56	71	8.2
C23	16	85		278	7.2	0.71	0.54	54	7.5
C24	17.2	60		254	5.9	0.68	0.51	59	8.7
C25	17.4	78		438	27.0	3.29	3.38	412	12.1
C39	16	88	3.9	327	8.3	0.86	1.30	135	14.4
C40	16	89	3.1	406	8.9	1.05	1.34	159	15.0
C41	16	80	3.9	275	12.5	1.23	1.47	145	12.8
Fasted									
F1	9.1	61		163	10.4	1.47	0.42	60	4.6
F2	9.1	61		123	4.7	0.64	0.37	47	8.0

* Figures after decimal points indicate number of days.

† Bodansky units per 100 cc. serum.

‡ Per lb. body weight.

§ All the dogs were sacrificed when they were still in good health.

** 5.9 units on 12th week, 8.1 on 14th and below 5 rest of the time.

(8.3 per cent and 8.9 per cent), and fatty infiltration was definite in two animals of this group (12.5 per cent and 27.0 per cent).

With one exception all livers from the protein-deficient dogs (without cholesterol) contained more lipids than those from the animal with an adequate protein intake, ranging from 6.6 per cent to 31.9 per cent with an average of 16.2 per cent. Cholesterol added to a protein deficient ration affected the liver in a more uniform

manner. A high degree of fatty infiltration was evident in all animals. The average total lipid content was 26.3 per cent, with a lower limit of 11.6 per cent and a maximum of 43.0 per cent. Fatty infiltration of the liver was not further increased by doubling the cholesterol intake (average 24.0 per cent—range 16.7 per cent to 35.5 per cent). There were 10.4 and 4.7 per cent lipids in the livers of the two fasted animals.

Total cholesterol in liver and its ratio to total lipids. The control dogs without cholesterol had 0.29 per cent cholesterol in their livers ranging from 0.26 per cent to 0.34 per cent. It constituted 4.2 per cent to 5.6 per cent of the total lipids

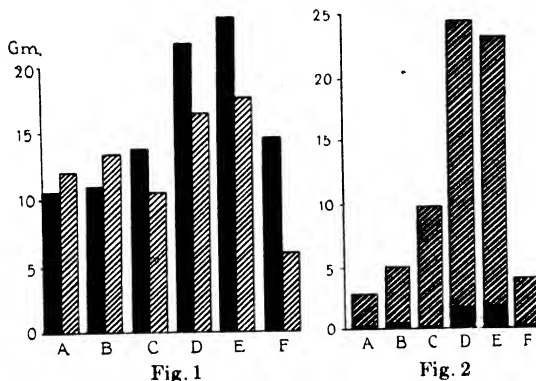


Fig. 1. Liver weight. Solid columns show per pound of final body weight. Cross-hatched columns show per pound of initial body weight. A—High-fat control diet (2.0 grams protein per lb. body weight). B—High-fat control diet (2.0 grams protein per lb. body weight) with cholesterol. C—High-fat protein-deficient diet. D—High-fat protein-deficient diet with cholesterol. E—High-fat protein-deficient diet with double dose of cholesterol. F—Fasting.

Fig. 2. Liver lipids. Cross hatched section of columns shows total lipids per pound of final body weight; solid section shows cholesterol per pound of final body weight. A—High-fat control diet (2.0 grams protein per lb. body weight). B—High-fat control diet (2.0 grams protein per lb. body weight) with cholesterol. C—High-fat protein-deficient diet. D—High-fat protein-deficient diet with cholesterol. E—High-fat protein-deficient diet with double dose of cholesterol. F—Fasting.

(average 4.9 per cent). Addition of cholesterol to the diet caused a very significant increase in its content in all the livers, varying from 0.51 per cent to 3.38 per cent with an average of 1.30 per cent. Its ratio to total lipids (average 11.2 per cent; range 7.5 to 14.4 per cent) was much higher than for those animals on the same diet without cholesterol.

There was no increase of cholesterol in the livers of the simple protein-deficient dogs (0.24 per cent to 0.38 per cent, average 0.27 per cent); because of the deposition of glycerides the cholesterol: lipids ratio decreased to 2.2 per cent (range 0.7 to 4.3 per cent).

Addition of cholesterol to the protein-deficient diet showed a similar but more

marked effect than in the control animals. The average cholesterol content was 1.80 per cent (range 0.59 to 3.5 per cent). These dogs, in contrast to the protein-deficient dogs without cholesterol, showed an increase in cholesterol relatively greater than in total lipids, and its ratio to the latter was 6.2 per cent, but because of the large amount of other lipids deposited, the ratio was not as high as when cholesterol was given with protein. Doubling the intake of cholesterol increased both the cholesterol and total lipid content of the liver, one dog had 4.09 per cent of cholesterol in the liver with a ratio to total lipids of 24.5 and cholesterol made up more than 1 per cent of fresh weight of two other livers in this group.

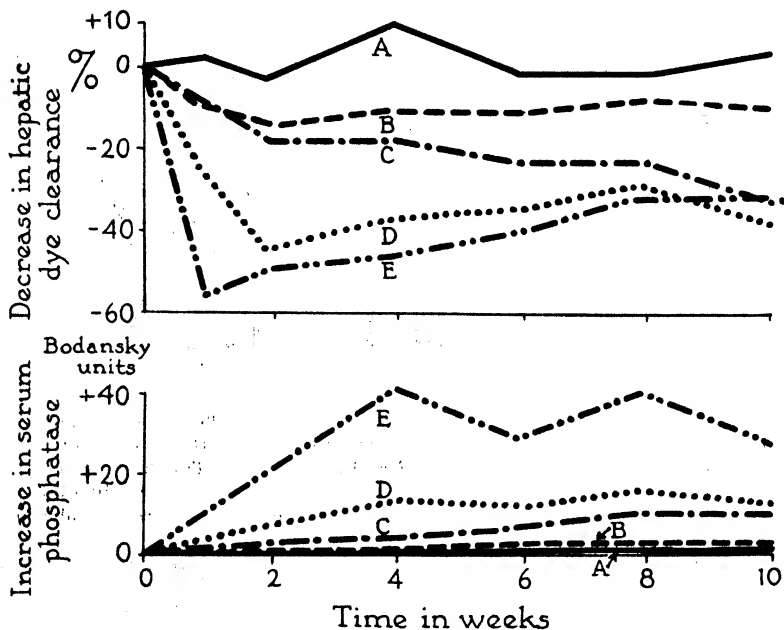


Fig. 3. Progressive changes in hepatic dye clearance and in serum phosphatase. A—High-fat control diet (2.0 grams protein per lb. body weight). B—High-fat control diet (2.0 grams protein per lb. body weight) with cholesterol. C—High-fat protein-deficient diet. D—High-fat protein-deficient diet with cholesterol. E—High-fat protein-deficient diet with double dose of cholesterol.

Expressing the amount of total lipids and cholesterol in the liver in terms of body weight gave similar results, as shown in figure 2.

Starvation did not have much influence on liver cholesterol.

Hepatic dye clearance and serum phosphatase. In tables 1A and 1B are shown the minimum values of Rose Bengal clearance in percentage and the maximum values of phosphatase in Bodansky units for individual dogs during the entire experimental period. With their initial values as zero the progressive changes of the liver function and serum phosphatase of various groups were plotted together against time (fig. 3).

Including some other normal data from other series, hepatic dye clearance for 82 normal dogs is 105 ± 9 per cent and serum phosphatase for 67 among this group is 1.5 ± 0.9 Bodansky units.

The average dye clearance and serum phosphatase of the control group without the cholesterol supplement were maintained in the normal range throughout the period of observation. Adding cholesterol to the control diet did not produce any effect on serum phosphatase, but it did depress the dye clearance. Six of seven dogs on several occasions had a clearance of less than 90 per cent. This effect occurred when they were on the ration for only two weeks. The dye clearance of these animals improved to some extent even though cholesterol was given daily.

Protein depletion caused a marked impairment in liver function. The rate of dye clearance was progressively reduced in dogs on the high-fat protein-deficient diet. This impairment can usually be demonstrated after 4-6 weeks of depletion, but occasionally occurs earlier or later. The lowest individual figures varied from 61 per cent to 1 per cent with a group average of 40 per cent. The serum phosphatase increased to five Bodansky units or greater after 2 to 4 weeks.

Dog C32 had an abrupt change between the eighth and tenth weeks. His liver function remained above 90 per cent till the eighth week but by the tenth week dropped to 1 per cent and was 41 per cent on the twelfth week. At the same time the serum phosphatase increased from 12.2 to 83.7 units and it was 35.0 units on the twelfth week.

Adding cholesterol to the high-fat protein-deficient diet seemed to produce more uniformly high values for serum phosphatase, especially in the group receiving a double dose of cholesterol.

All thirteen protein-deficient dogs fed cholesterol showed a marked initial fall in the rate of dye clearance. The loss of this function was striking and could be detected in the first week, in fact after only 4 days on the regimen. The highest clearance among the 7 dogs after 4 days was 88 per cent, while the lowest was 44 per cent. The function in the second week of all 13 animals ranged from 63 per cent to 35 per cent. Doubling the cholesterol intake had a further reducing effect on the dye clearance, three dogs had only 74 per cent, 47 per cent, and 44 per cent respectively in the first week. The protein-deficient dogs without cholesterol usually showed no or only a little change at the end of two weeks. Their clearance rate at this time ranged from 109 per cent to 65 per cent; on an average they still retained 82 per cent of the normal function. However, the maximum effect of cholesterol was not a permanent one, the dye clearance rate rose somewhat in subsequent weeks, approaching the values obtained on the simple protein-deficient dogs by the tenth week.

Fasting animals showed a progressive but less marked decrease in hepatic dye clearance, the lowest figure being 61 per cent. Their serum phosphatase values were found to be normal on two occasions.

Part II—One Week Experiment. All the results of the one week experiment may be found in table 2. All animals consumed their ration soon after it was given.

There was a definite decrease in hepatic dye clearance as early as the fourth day, when cholesterol was given without protein. Their dye clearance was depressed to as little as 36 per cent and 15 per cent in 7 days. There was no definite evidence of such depression within 7 days in dogs on the diets with protein and with cholesterol nor in dogs on the protein-deficient diet without cholesterol. Elevation of serum phosphatase was quite definite by the fourth day in cholest-

TABLE 2

DOGS	HEPATIC DYE CLEARANCE				SERUM PHOSPHATASE				LIVER					
	(number of days)				(number of days)				Total lipids		Cholesterol		'sterol lipids	
	0	2	4	7	0	2	4	7	Conc.	Per lb.†	Conc.	Per lb.†		
High-fat protein-deficient diet														
	per cent	per cent	per cent	per cent	units*	units*	units*	units*	per cent	gram	per cent	mgm.	per cent	
C51	123	117	102	111	0.5	1.2	1.7	3.5	5.2	0.68	0.22	29	4.3	
C66	105	96	83	90	2.0	0.3	4.6	3.7	4.7	0.62	0.30	40	6.3	
High-fat protein-deficient diet + cholesterol														
C67	124	99	84	36	2.4	2.5	7.5	14.8	8.9	1.51	1.55	260	17.3	
C68	100	96	67	50	1.8	2.2	5.7	11.9	9.3	1.22	1.37	180	14.8	
High-fat protein-deficient diet + 5 X cholesterol														
C47	121	115	75	15	1.6	4.1	12.8	27.1	14.0	1.61	2.71	312	19.3	
C49	131	123	98	81	3.5	5.8	6.4	5.6	9.0	1.34	1.50	218	16.6	
High-fat 25% protein (2.0 grams per lb.) diet + cholesterol														
C69	117		86	105	1.7	1.7	1.7	1.5	6.6	0.78	0.47	56	7.1	
C70	90	93	86	85	0.8	0.8	0.8	1.6	6.4	0.68	0.61	64	9.4	
High-fat 25% protein (2.0 grams per lb.) diet + 5 X cholesterol														
C50	105	91	95	84	0.9	1.2	1.2	2.7	7.4	1.01	1.13	155	15.3	
C65	116	100	95	95	2.6	1.0	2.9	1.5	6.4	0.86	1.19	160	18.8	
High-fat 40% protein (3.2 grams per lb.) diet + cholesterol														
C71	111	112	101	98	1.2	1.8	1.2	1.8	4.4	0.66	0.41	61	9.2	
C72	104	90	91	91	1.7	0.9	1.9	1.9	4.2	0.79	0.32	41	7.6	

* Bodansky units per 100 cc. serum.

† Per lb. final body weight.

terol fed protein-deficient dogs. It is impossible to decide from so few animals whether there was a greater rise in serum phosphatase with larger doses of cholesterol. The elevation of serum phosphatase in the simple protein-deficient dogs did not go beyond the normal limit in one week. Little or no rise of this enzyme occurred in blood when protein was given with cholesterol.

None of the livers, whether with or without cholesterol or with or without

protein, were enlarged after 7 days. Total wet weight as well as per pound body weight were all within normal limits.

Ingested cholesterol and protein depletion together for 7 days caused an increase in total liver lipids which was directly related to the cholesterol intake. Total liver lipids resulting from 0.1 gram cholesterol per pound body weight were 8.9 per cent and 9.0 per cent. Those receiving 0.5 gram cholesterol per pound were 9.3 per cent and 14.0 per cent. There was practically no increase in total liver lipids when cholesterol was given with protein for 7 days. Only the concentration of total lipids in the liver of dog C50, which was fed the high-fat 25 per cent protein diet with 0.5 gram cholesterol per pound, was slightly higher—7.6 per cent. Protein depletion for one week had no effect on liver lipids.

Administration of cholesterol for a week affected the liver cholesterol to a greater or less degree depending upon whether or not protein was given. The greater the protein intake the less was the accumulation of cholesterol in the liver. The animals on the protein-deficient diet with 0.1 gram cholesterol per pound body weight had 1.37 and 1.55 per cent cholesterol in their livers. Those with 0.5 gram cholesterol had 1.50 and 2.71 per cent. The liver cholesterol was elevated to 0.47 and 0.61 per cent in the animals with 2 grams casein per pound by including 0.1 gram cholesterol per pound body weight in the diet. Five times this amount of cholesterol caused 1.13 and 1.19 per cent cholesterol in the liver.

Increasing the protein intake to 3.2 grams per pound did not entirely prevent the accumulation of cholesterol. The group on this level of protein intake with 0.1 gram cholesterol per pound had 0.32 and 0.41 per cent of cholesterol in the liver, which was slightly higher than normal (0.29 per cent). The high-fat protein-deficient diet without cholesterol had no effect on liver cholesterol in 7 days.

DISCUSSION. *Increase in liver weight and in total amount of liver lipids.* Enlargement of the fatty livers can largely be accounted for by the extra quantity of lipids. It was impossible to ascertain the exact size and lipid content of these livers at the beginning of the experiment. It is an approximation to assume that the livers of the protein-deficient dogs originally were the same size and had the same fat content per pound body weight as were found in the animals fed on the high-fat control diet which supplied them with an adequate amount of protein. On this basis, the increase in liver weight and in total liver lipids in various groups during the entire experiment, were calculated and are shown in figure 4. The increase in lipids accounted for from 46 per cent to more than 100 per cent of the enlargement. The decrease in the total amount of liver fat accounted for only 8 per cent of the total shrinkage of this organ in starvation.

Hepatic dye clearance and serum phosphatase. Hough and Freeman (34) demonstrated an inverse relation between the increase in serum phosphatase and the decrease in hepatic dye clearance of protein-deficient dogs. In general, protein depletion in this experiment had the same effect. However, the sharp drop in hepatic clearance in four days, without a proportional rise of serum phosphatase, when an extensive amount of cholesterol was given with a high-fat protein-deficient diet, needs some further explanation. There might be a temporary block-

ade by cholesterol of the excretory pathway of the dye. This blockade could be produced by saturation of the Kupffer cells of the liver with cholesterol or by deposition of cholesterol within the cells that make up the hepatic parenchyma. Serum phosphatase is not excreted by the liver, according to the work done in this laboratory. In the experiment of Freeman and Chen (42) phosphatase remained above normal for several days when normal dogs were transfused with blood from dogs with a high serum phosphatase caused by an obstructive jaundice. Evidence for the belief that phosphatase in bile originates in the liver, and

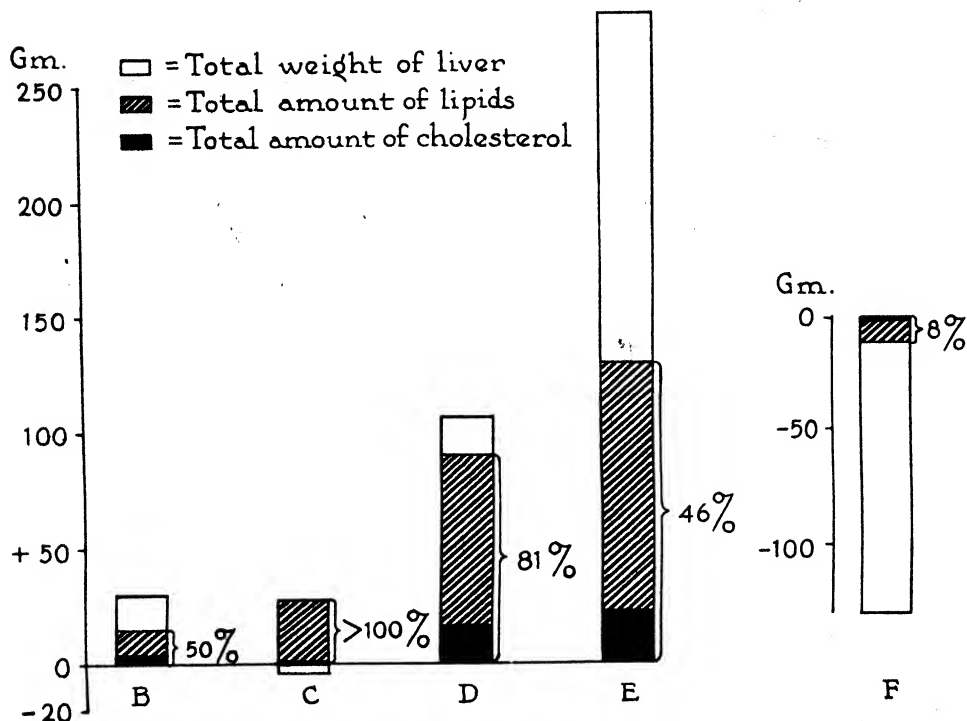


Fig. 4. Calculated changes in liver. B—High-fat control diet (2.0 grams protein per lb. body weight) with cholesterol. C—High-fat protein-deficient diet. D—High-fat protein-deficient diet with cholesterol. E—High-fat protein-deficient diet with double dose of cholesterol. F—Fasting.

that its increase in the blood stream is caused by obstruction or changes in permeability, has been given by Freeman, Chen and Ivy (43). The relatively greater effect of cholesterol upon dye clearance supports the view that serum phosphatase is not excreted by the liver; if it is, the excretory mechanism must differ from that of a dye or bilirubin. The increase in dye clearance subsequently observed in protein-deficient dogs fed cholesterol may have been due to reduced cholesterol absorption secondary to lessened bile formation by the protein-deficient animals (44, 45).

Liver lipids. Rubin, Present and Ralli (46) found the average lipid content to be 4.81 per cent and cholesterol to be 0.255 per cent in the livers of 33 normal dogs. Chaikoff and Connor (47) obtained fatty livers in 4 dogs fed 10 grams of lard and 7 grams of lean meat per kilo body weight for 138 to 386 days. In the present study, the average liver lipids of seven dogs on the high-fat adequate protein diet was 6.2 per cent lipids and 0.29 per cent cholesterol. There were two instances in which dogs on the control diet with cholesterol had fatty livers and only slightly reduced dye clearance. However, among the high-fat protein-deficient dogs there was no instance of a fatty liver without a definite reduction in the rate of Rose Bengal clearance.

Though prolonged protein-deficiency causes a fatty liver, yet there was no evidence of such a change by the end of the first week. Evidently the protein store of the body must be more depleted before fat accumulates in the liver; this finding is in contrast to the effect of cholesterol.

The effect of cholesterol. The literature contains no information concerning the effect of cholesterol on the liver of dogs. The general impression seems to be that dogs are resistant to cholesterol, at least much more resistant than rabbits or rats. In the present study it has been shown that cholesterol influenced the dye clearance and fattiness of the liver of some dogs even with an adequate protein intake. Protein-deficient dogs were all adversely affected by cholesterol so far as liver function, serum phosphatase and fattiness of the liver are concerned. However, the incidence of arteriosclerosis and peptic ulcer was no greater in the cholesterol-fed animals.

The amount of cholesterol that accumulated in the liver in one week was, in some of the cholesterol-fed dogs, as high as ten times the average normal content. There was an increase in total lipids also, but it is evident that deposition of fatty acids at this early period was not the result of protein deficiency but was due to formation of esters of cholesterol. The high ratio of cholesterol to total lipids indicates that a greater part of the increase of liver cholesterol was in the free form.

It was not possible to follow the change of concentration of cholesterol in the liver of the deficient animals at all times, but it is reasonable to assume that the amount of total cholesterol in the liver increased continuously after as well as during the first two weeks, when the liver function dropped sharply. It is probable that there was a change in the distribution in the different fractions of total cholesterol as the experiment progressed. Fractional analysis of cholesterol in the livers of choline-deficient rats by Loizides (22) indicated a great increase in its ester and only a little rise in its free form. This result was obtained after the effect of cholesterol was more advanced, and perhaps the picture was different earlier. Free cholesterol may exert a more harmful effect on the liver or a more effective blockade of the route of dye excretion than does its esters. The liver may only be released from the adverse action of free cholesterol by converting it into esters. A similar suggestion was made by Best, Channon and Ridout (40), who found a marked increase of free cholesterol in the livers of choline-deficient rats fed cholesterol.

The lipotropic action of protein. The lipotropic action of protein in dogs was

clearly demonstrated by the absence of fatty infiltration of the liver in the animals with an intake of 2.0 grams of casein per pound daily.

The action of protein in relieving the adverse effect of cholesterol is clearly demonstrated in the one week experiment. By including 25 per cent casein in the diet, cholesterol in the liver was reduced from 1.55 and 1.37 per cent to 0.61 and 0.47 per cent. Further increase of the protein level to 40 per cent reduced liver cholesterol still further, to 0.41 and 0.32 per cent (table 2)—values that are slightly above or within the normal limits (table 1B). Thus 2.0 grams casein per pound body weight is not enough for the prevention of cholesterol fatty liver in dogs. It is apparent from the results of these experiments that a high intake of protein is necessary to prevent the accumulation of cholesterol in the liver of dogs fed cholesterol. That this should be the case suggests that an interrelation exists between the catabolism of cholesterol and protein.

SUMMARY

1. Fatty liver was produced in dogs maintained on a 33 per cent fat protein-deficient diet for 10 to 16 weeks. There was no fatty infiltration at the end of the first week on this diet.

2. Added cholesterol caused a uniformly higher total lipid and cholesterol content of the liver of protein-deficient dog. Lipid deposition was evident after seven days of cholesterol feeding. The evidence indicates that more cholesterol was in the free form than is normally the case.

3. Two grams of casein per pound body weight daily can prevent fatty infiltration of the liver in dogs on a high fat diet for sixteen weeks, but this protein intake is not sufficient to prevent "cholesterol" fatty livers.

4. Protein depletion impairs hepatic dye clearance and causes elevation of serum phosphatase in dogs.

5. Adding cholesterol to the high-fat protein-deficient diet resulted in an abrupt drop in dye clearance at the beginning of the experiment, and a uniformly higher elevation of serum phosphatase than that caused by simple protein deficiency.

6. There is generally an inverse relationship between serum phosphatase and Rose Bengal clearance, but initially there was a disproportionately sharp drop of dye clearance in the cholesterol-fed protein-deficient dogs.

7. Irrespective of the diet or duration of the experiment, all the dogs with increased liver lipids or cholesterol had a reduced dye clearance. However, the degree of impairment in dye clearance was not necessarily proportional to the fattiness or cholesterol content of the liver.

8. Enlargement of the fatty liver was largely or entirely accounted for, according to calculations, by the increase in lipids.

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EXPERIMENTAL LIPEMIA AND HYPERCHOLESTEROLEMIA BY PROTEIN DEPLETION AND BY CHOLESTEROL FEEDING IN DOGS

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Alimentary lipemia after feeding fat to dogs was reported by Bloor in 1914 (1). An occasional high post-absorptive lipid level caused by overfeeding was reported by the same author (2). Further, he (3) showed how the fat content of the diet influences the blood lipids, though the effect of diet was more marked in rabbits than in dogs. Entenman and Chaikoff (4) found that overnutrition had a tendency to cause blood fatty acids to increase, but there was little or no rise in total cholesterol.

Page, Farr and Weech (5), in correlating hypoproteinemia and lipemia, found that there was some elevation in blood lipids when dogs were depleted of protein, but this result was not consistent. Bollmann and his associates (6, 7) reported that sustained post-absorptive lipemia was produced on a high fat diet only when lecithin was given.

Attempts to produce hypercholesterolemia in dogs by cholesterol feeding have led to contradictory results (8). Steiner and Domanski (9) found a sustained increase of blood cholesterol in dogs as well as in men fed with egg yolk powder which contained 8 per cent cholesterol. Flock et al. (6) and Corwin (8) were unable to further increase the lipemia and hypercholesterolemia by adding cholesterol to a high fat diet with lecithin.

The present study was intended to contribute further information concerning the effect of diet, particularly protein deficiency and cholesterol ingestion, upon the post-absorptive fat content of the blood; also to ascertain to what extent the blood lipid level is related to the degree of fat accumulation in the liver or to alterations in hepatic dye clearance or serum phosphatase. The last mentioned data are contained in the preceding article (10).

PROCEDURE. Seven adult male dogs were fed with a high-fat (33 per cent) protein-deficient diet (practically free from protein except that contained in yeast, which constituted 5 per cent of the diet), while another 9 had 0.1 gram cholesterol per pound body weight daily in addition to the above diet (given in gelatin capsules just before the meal). The composition of the diet, amount of the food fed, Rose Bengal dye clearance, serum phosphatase level and liver lipids in these animals were described in another communication (10). In this report only serum lipids will be considered.

Blood specimens were obtained while the animals were in a post-absorptive state (36-40 hrs. after last meal) at the beginning of the experiment, and every other week thereafter, for the determination of total serum fatty acids and cholesterol. Serums were extracted with ether and alcohol mixture by Bloor's method (11). Total fatty acids were determined according to the method of

Smith and Kik (12). Cholesterol was determined according to the method of Hoffman (13).

As controls, a group of four dogs were kept on an isocaloric diet which supplied 2.0 grams casein daily per pound body weight with the same percentage of lard. Another group of five dogs were fed with the same control diet and in addition 0.1 gram cholesterol per pound body weight daily.

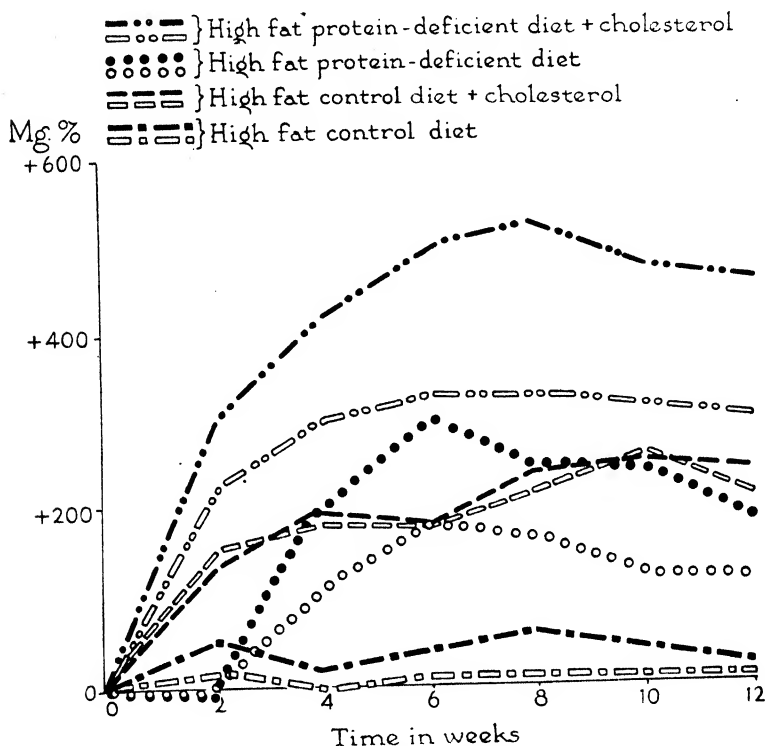


Fig. 1. Increase in serum total fatty acids and total cholesterol. Closed pattern = total fatty acids. Open pattern = total cholesterol.

RESULTS. The initial values of these twenty-five dogs varied from 262 to 629 mgm. of total fatty acids and from 107 to 295 mgm. of total cholesterol per 100 cc. of serum. Including some normal values from other series of experiments, average serum total fatty acids were 453 ± 88 mgm. per cent for 32 dogs and total cholesterol was 183 ± 41 mgm. per cent for 60 dogs.

The average increase in total fatty acids and cholesterol of various groups, with their respective initial values as zero, are shown in figure 1. The relation between total fatty acids and cholesterol of individual samples of blood is expressed by the lipemic coefficient (cholesterol \div total fatty acids $\times 100$) (14). A decrease in

lipemic coefficient indicates that the total fatty acids increased relatively more than cholesterol. A relatively higher rise in cholesterol is evidenced by an increase in this figure. Average alterations of the lipemic coefficient in different groups throughout the entire experiment are shown in figure 2.

The high-fat content of the control diet, which provided 2 grams of casein per pound body weight daily, had some effect in raising the post-absorptive level of lipids in the blood of dogs, though the elevation is slight and the result was not consistent in all animals. There were maximum increases of 117, 86 and 229 mgm. per cent in the serum total fatty acids of dogs C16, C28 and C29 respectively and only three out of twenty-four individual observations fell below their corresponding initial values. On the other hand, dog C30 had only 33 mgm. per cent maximum increase in serum total fatty acids, and values obtained on the diet were below the initial value most of the time.

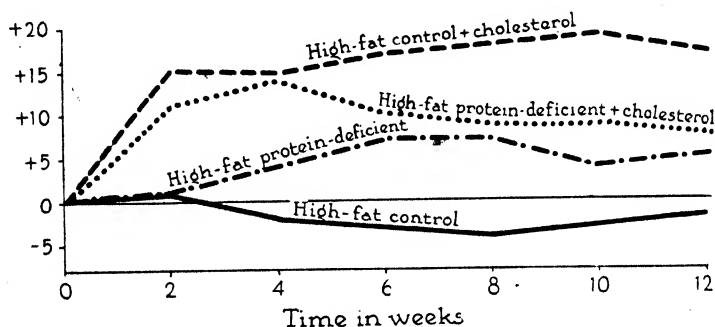


Fig. 2. Alteration in lipemic coefficient $\left(\frac{\text{Cholesterol}}{\text{Total fatty acids}} \times 100 \right)$

The high-fat content in the control diet had little effect on serum cholesterol. The maximum increase ranged from 38 to 67 mgm. per cent. Because of the increase of total fatty acids, a decrease in lipemic coefficient resulted.

When cholesterol (0.1 gram per pound body weight) was given with the high-fat control diet, there was an increase in both serum cholesterol and total fatty acid levels. The rise was much more than could be considered as a normal fluctuation. The average maximum increase in this group was 261 mgm. per cent in cholesterol and 316 mgm. per cent in total fatty acids as compared to 44 mgm. per cent and 141 mgm. per cent respectively in the group on the same diet without the cholesterol supplement. The relatively greater increase in cholesterol resulted in a higher lipemic coefficient.

Protein deficiency definitely caused lipemia and hypercholesterolemia. Fasting blood specimens with a milky appearance were frequently obtained from this group. Two of these dogs had a serum fatty acid level of 1126 and 1061 mgm. (C1 and C22 respectively). The average maximum increase for the group above their initial values was 399 mgm. per cent. Serum cholesterol was raised, the average maximum was 208 mgm. per cent. The lipemic coefficient was elevated.

Excessive cholesterol feeding to protein-deficient dogs had a marked effect. Gross lipemia was seen most often among this group. The maximum increase in serum total fatty acids and cholesterol was much higher than for any other group. Dog C2 had 2110 mgm. of total fatty acids and 1400 mgm. cholesterol per 100 cc. serum after 10 weeks on this regimen. Another dog 1T had 2016 mgm. per cent of fatty acids after 6 weeks and 1774 mgm. per cent of cholesterol after 8 weeks on the experimental diet. The average maximum increase above the initial values for this group was 679 mgm. per cent of total fatty acids and 456 mgm. per cent of cholesterol. The lipemic coefficient was higher than the group on the same diet without cholesterol, but it was lower than the group with the same amount of the supplement on the control diet.

The increase in serum lipids was detectable from the first determination after the animals were on the protein-deficient diet with cholesterol. The same is true of the control diet with added cholesterol. The increase in the protein-deficient group without the cholesterol supplement came later. The highest values usually occurred between the sixth and tenth weeks on the experimental diets.

DISCUSSION. There was a consistent increase in the fasting level of the total serum fatty acids in three of four dogs fed the high fat control diet but no corresponding increase in serum cholesterol. The fact that the fasting blood lipid level of an individual is normally maintained within narrow limits was demonstrated by Terroine (15), Mayer and Schaeffer (16), Bang (17) and Bloor (18). Terroine (15) found that the absolute values for both cholesterol and fatty acids might vary widely from dog to dog, but those values for any given animal varies but little from time to time in the 36 hour post-absorptive state. Their results lend support to the belief that the increase in total serum fatty acids observed on the high-fat control diet cannot be considered merely as a normal fluctuation. Under our experimental conditions it seems that extra lecithin is not necessary for the elevation of blood lipids on a high fat diet. This finding is different from that of Flock et al. (6). The lack of an increase of serum cholesterol on the high-fat diet in the presence of an increased total serum fatty acids is a similar finding to that reported by Entenman and Chaikoff (4) in their study of overnutrition.

The very significant increase both in cholesterol and in total fatty acids seen in all seven protein depleted dogs clearly demonstrated that lipemia and hypercholesterolemia were constant accompaniments of protein depletion on this experimental diet. The different results reported by Weech and his associates (5) may have been due to differences in protein and fat levels in the diet.

Exogenous cholesterol had the same effect on dogs as has been found in rabbits (19, 20). This is evidenced by the definite elevation in blood cholesterol (more than 100 per cent above the initial value) even in the group with an adequate protein-intake. The average maximum increase in serum cholesterol in the protein-deficient group was 208 mgm. per cent. That in the group on the same diet with added cholesterol was 457 mgm. per cent. One dog C2 of the latter group had an increase to a level which is almost ten times as high as its initial value.

Cholesterol, as well as phosphatides, is concerned in the transport of fatty acids in the animal body (21, 22); 55 per cent of serum fatty acids are in combination

with cholesterol (23). Approximately 70 per cent of serum cholesterol is present as fatty acid esters. It is not surprising to find an increase of one accompanied by an elevation of the other. Therefore an increase of cholesterol will indicate that an increase in fatty acids is also occurring, though the elevations may not be proportional.

There was a relatively greater increase of serum cholesterol than of fatty acid in protein-deficient animals as well as in those that received extra cholesterol. Elevation of the lipemic coefficient was not so high as in two groups fed extra cholesterol, yet it was definite as shown by the average values (see fig. 2) and by most of the individual observations. There was a progressive increase of this coefficient during the first few weeks. It seemed that a relative as well as an absolute cholesterol increase accompanies this type of lipemia. The source of extra cholesterol in the circulation is not known. It may be dietary in origin, and its accumulation in the blood may be the result of impaired catabolism caused by protein deficiency (10).

TABLE 1

	C20				C32			
	Weeks on experiment				Weeks on experiment			
	6	8	10	12	6	8	10	12
Serum total fatty acids (mgm. %).	773	440	184	34	750	231	395	96
Serum total cholesterol (mgm. %).	416	233	88	76	244	69	135	10
Rose Bengal clearance (%).	86	40	36	36	90	90	1	41
Serum phosphatase (units).					10.9	12.2	83.7	35.0
Liver lipids—conc. (%)	31.9				14.4			
—total amt. (gm.)	105				54			

In all animals, serum lipids had a tendency to decline eventually, but some degree of lipemia and hypercholesterolemia persisted. Exceptions to this were dogs C20 and C32 of the protein-deficient group; their blood lipids fell far below the normal values. Their total serum fatty acids and cholesterol values together with their hepatic dye clearance rate and serum phosphatase level during this period are shown in table 1. These two dogs died at the end of the twelfth week; their liver lipid values are also included in the table. All blood lipid determinations were made in duplicate, and the values for C32 in the eighth week were verified on another specimen drawn a few days later. There is no apparent explanation for these abrupt changes. Dog C20 had the highest percentage as well as highest amount of total lipids and of cholesterol in the liver among the group of 7 protein-deficient dogs. The concentration of liver lipids and cholesterol, though, were not the next highest for dog C32, yet the total amount of these substances stood second among the whole group, because of the relatively large size of the liver. These observations suggest that there may be

some relation between the sudden alterations in blood fats, liver function and the high degree of fatty infiltration of the liver.

Though both lipemia and fatty liver are the result of protein-depletion and cholesterol ingestion, yet no definite correlation could be found. All 5 dogs on the high-fat control diet with cholesterol had lipemia, while only two of their livers contained more fat than those on the same diet without the supplement. To illustrate: dog C1 had a maximum increase of 662 mgm. per cent in serum total fatty acids, but his liver had only 12 per cent of lipids; while the maximum increase in total serum fatty acids of dog C19 was only 152 mgm. per cent but there was 25.8 per cent lipids in its liver. McKibbin, Thayer and Stare (24) were unable to find any proportional relationship between liver lipids and plasma cholesterol in puppies on a choline-deficient diet.

Besides what has been mentioned above, no definite correlation could be made between the maximum increase in serum lipids and the lowest depression of hepatic dye clearance and the highest elevation in serum phosphatase. A study of the individual figures gave the same impression.

SUMMARY

1. The dogs fed the high-fat (33 per cent) control diet with 25 per cent of casein had some increase in serum total fatty acids but not in cholesterol.
2. An increase of total serum fatty acids and cholesterol resulted in dogs fed the high-fat control diet with added cholesterol and in dogs fed the high-fat protein-deficient diet with or without cholesterol.
3. The increase of cholesterol was proportionally more than that of total fatty acids in all three groups as indicated by the elevation of the lipemic coefficient.
4. Though lipemia, fatty liver, decrease of hepatic dye clearance and increase of serum phosphatase were all accompaniments of protein-deficiency and cholesterol feeding, yet no definite correlation was demonstrated.

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THE IMPORTANCE OF EXOGENOUS FAT IN FATTY INFILTRATION OF THE LIVER OF DOGS

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A high-fat protein-deficient diet with or without cholesterol causes fatty infiltration of the liver, but the degree of fattiness varies greatly (1). As protein-depletion and ingestion of cholesterol impair liver function, presumably the production of bile salts and the flow of bile into the intestine are also reduced. Smith and Whipple (2) proved that food protein determines the level of the exogenous bile salt output. Kocour and Ivy (3) demonstrated the potency of meat as a stimulant of bile-volume output. Thus, bile salts may influence fat infiltration of the liver by virtue of their effect upon fat absorption in the protein-deficient dog.

Fatty livers have been produced in rats fed a diet free from fat (4). Dietary carbohydrate was shown, by Barrett, Best and Ridout (5), to be the source of liver fat in mice fed a lipotropic-deficient diet low in fat. Hansen and Wiese (6) and Miller and Hansen (7) showed that fatty acids can be synthesized by dogs on a low fat diet. How much the development of fatty livers in dogs depends upon exogenous fat has not been studied.

Our previous communications (1, 8) have shown that a high-fat protein-deficient diet influences hepatic dye clearance, serum phosphatase, and total serum fatty acids and cholesterol, and also that ingested cholesterol enhances the effects of protein-deficiency.

The following study was undertaken to determine to what extent exogenous fat and bile salts influence liver function, serum phosphatase, blood lipids and fattiness of the liver of dogs maintained on a protein-deficient diet.

PROCEDURE. The diets used in this study are shown in table 1. The high-fat (33 per cent) protein-deficient diet was the same as that used in our previous studies (1, 8). All the diets are practically free from protein except for that in the yeast, and all are isocaloric with respect to vitamins, minerals and proteins. All dogs were fed 40 Calories per pound body weight daily except Sundays.

Adult male dogs, after being kept for observation in the laboratory for two or more weeks, were used for the experiment. Before the experiment, they were fed Pard or dehydrated Pard¹.

A. The effect of bile salts. Six dogs were used in the experiment. Three of them were fed the high-fat protein-deficient diet and another three had 0.1 gram cholesterol² per pound body weight daily in addition to the above diet. All were

¹ Dog food prepared by Swift and Co., Chicago.

² Dr. David Klein, director of the Wilson Laboratories, Chicago, kindly supplied us with the cholesterol and bile salts used in this study.

given 2.75 grams Wilson's desiccated ox-bile salts in gelatin capsules² just prior to feeding.

Rose Bengal clearance and serum phosphatase were determined before the experiment, at the end of the first and second weeks, and every two weeks thereafter. Blood specimens were drawn for lipid estimations 36 hours after feeding. In the group fed the cholesterol supplement, both total serum fatty acids and cholesterol determinations were made; in the group without cholesterol, only the latter was determined.

The dogs with cholesterol were sacrificed when they were moribund. The animals without the supplement were sacrificed at the end of the twelfth week. Autopsies were all done immediately after death, and the livers were weighed and analyzed for total lipids and cholesterol. These procedures have been described previously (1, 8).

TABLE 1
Protein-deficient diet of varying fat content

	2% FAT	7% FAT	15% FAT	HIGH FAT (33%)
Sucrose (or dextrin).....	88.5%	83.5%	74%	55%
Lard.....	2	7	15	33
Dried brewers' yeast.....	4	4	4.5	5
Cellophane.....	4	4	4.5	5
Wesson's salt mixture (11).....	1.5	1.5	2	2
Percomorph oil.....	8 drops per kgm.	8 drops per kgm.	9 drops per kgm.	10 drops per kgm.
Amt. fed per lb. body weight.....	11 gm.	10.5 gm.	9.5 gm.	8.0 gm.
Calories supplied by fat.....	4.8%	15.9%	31.3%	57.4%

B. The effect of dietary fat level. Twelve dogs were used in this experiment; 6 on the 2 per cent fat diet, and 3 each on the 7 per cent fat and 15 per cent fat diets. Rose Bengal clearance, serum phosphatase and serum cholesterol were determined before the experiment and at two week intervals thereafter. Three of the group on the 2 per cent fat diet were sacrificed at the end of the twelfth week and all others were sacrificed when they were moribund. Autopsies were done and livers were weighed and analyzed as described previously.

RESULTS. *A. The effect of bile salts.* In table 2 are included survival period, weight loss, maximum alterations in Rose Bengal clearance and serum phosphatase, and the results of liver analysis in these dogs. The progressive changes of hepatic dye clearance and serum phosphatase during the first ten weeks are shown in figure 1. In the table and the graph the corresponding data from previous studies (1) without the bile salt supplement are included for comparison.

The survival period of the dogs fed on the protein-deficient diet and cholesterol with bile salts was not significantly different from those animals without bile salts. Among the three, one lived only 6 weeks, but the other two survived 12

and 14 weeks. Those without bile salt supplement survived 10 to 18 weeks, average $13\frac{1}{2}$ weeks. All three dogs fed on the protein-deficient diet with bile salts were still alive but in poor condition at the end of the twelfth week, and consequently they were sacrificed. The group on the same diet without bile salts survived 12 to 17 weeks, average $14\frac{1}{2}$ weeks. Dogs fed bile salts lost their appe-

TABLE 2

DOGS	SURVIVAL PERIOD	MINIMUM DYE CLEARANCE	MAXIMUM P-TASE	LIVER					
				Weight	Total lipids		Cholesterol		'sterol lipids
					Conc.	Per lb. ‡	Conc.	Per lb. ‡	
High-fat protein-deficient diet + bile salts									
	<i>weeks*</i>	<i>per cent</i>	<i>units†</i>	<i>gram</i>	<i>per cent</i>	<i>gram</i>	<i>per cent</i>	<i>milligram</i>	<i>per cent</i>
C62	12+	47	35.0	324	24.8	4.95	0.44	88	1.8
C63	12+	68	6.2	355	17.9	3.53	0.39	76	2.2
C64	12+	60	8.3	346	14.5	2.19	0.38	57	2.6
Ave.....		58	16.5	342	19.1	3.56	0.30	74	2.2
High-fat protein-deficient diet									
Range	12-17.2	1-61	10.5-83.7	198-372	6.6-31.9	0.76-6.00	0.23-0.38	29-62	0.7-4.3
Ave.....	14.3	46	25.0	266	16.2	2.49	0.27	38	2.2
High-fat protein-deficient diet + cholesterol + bile salts									
C44	12	39	25.8	482	28.9	7.10	2.41	588	8.3
C45	6	39	23.6	347	29.3	7.68	1.35	354	4.6
C46	14	48	29.1	272	35.4	6.41	1.52	276	4.3
Ave.....	10.5	42	26.2	367	31.2	7.06	1.76	406	5.7
High-fat protein-deficient diet + cholesterol									
Range	10-18	35-58	4.3-30.8	150-602	11.6-43.0	1.39-14.79	0.59-3.50	98-846	3.3-12.2
Ave.....	13.4	46	19.4	351	26.3	6.28	1.80	445	6.2

* Figures after decimal points indicate number of days.

† Phosphatase in Bodansky units per 100 cc. serum.

‡ Per lb. body weight.

tite earlier than the animals on the same diet without it. Except one animal which lost $12\frac{1}{2}$ pounds, changes in body weight were similar whether or not bile salts were given.

Early in the experiment there was a more striking depression of hepatic dye clearance in the dogs receiving bile salts, but later in the experiment this difference ceased to exist. Of determinations done in the first week, four pro-

tein-deficient dogs were 100, 96, 86, 85 per cent; two dogs fed the same diet with bile salts were 88, 68 per cent. The cholesterol groups all had an abrupt drop in dye clearance which was particularly marked in the animals with bile salts. Dye clearances at the end of the first week were 88, 82, 72, 71 per cent for those not fed bile salts and 66, 58, 43 per cent for those fed with this supplement.

The blood lipid content was not definitely influenced by feeding bile salts. Dogs fed bile salts had a lower average blood lipid level than the unsupplemented protein-deficient groups. The variability of the values for all these groups was too great to make this difference significant.

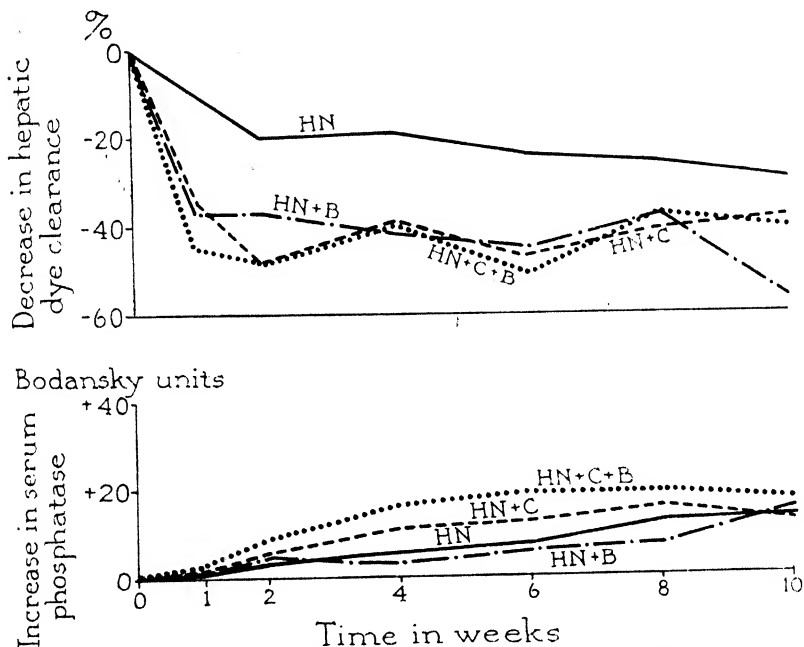


Fig. 1. Effect of bile salts. HN = high-fat protein-deficient diet; C = cholesterol; B = bile salts.

Liver analyses suggest that the percentages of lipids in the liver are more uniformly high in animals fed bile salts. The degree of fatty infiltration of the liver in dogs fed the protein-deficient diet with or without cholesterol varied greatly. Total lipids ranged 6.6 to 31.9 and averaged 16.2 per cent and cholesterol ranged 0.23 to 0.38 and averaged 0.27 per cent in protein-deficiency without cholesterol. Those with cholesterol ranged 11.6 to 43.0 and averaged 26.3 per cent in total lipids and ranged from 0.59 to 3.50 (average 1.80) per cent in cholesterol. With bile salts added to the ration, protein-deficient animals had 24.8, 17.9, and 14.5 per cent of total lipids and 0.44, 0.39, and 0.38 per cent of cholesterol; and those on the same deficient diet with cholesterol had 35.4, 29.3, 28.7 per cent and 2.41, 1.52, 1.35 per cent, respectively.

B. The effect of dietary fat level. The survival period, the change in body weight, maximum alterations in Rose Bengal clearance and serum phosphatase, and the results of liver analysis are included in table 3. The progressive change in hepatic dye clearance and serum phosphatase during the first ten weeks are

TABLE 3

DOGS	SURVIVAL PERIOD	MINIMUM DYE CLEARANCE	MAXIMUM P-TASE	LIVER					
				Weight	Total lipids		Cholesterol		'sterol lipids
					Conc.	Perl b.†	Conc.	Per lb.†	
2% fat protein-deficient diet									
C59	weeks*	per cent	units†	gram	per cent	gram	per cent	milligram	per cent
C60	12+			537	4.0	0.74	0.30	57	7.4
C61	12+			663	2.4	0.64	0.18	48	7.5
	12+			405	3.2	0.67	0.23	47	6.9
Ave.....				535	3.2	0.68	0.24	51	7.3
C73	21	70	12.4	221	3.4	0.54	0.30	48	8.8
C74	17.2	51	11.6	277	6.9	0.87	0.32	40	4.6
C75	15	51	13.7	198	8.3	1.24	0.42	62	5.0
Ave.....	17.5	57	12.6	232	6.2	0.88	0.35	50	6.1
7% fat protein-deficient diet									
C76	14	83	10.1	373	4.6	0.74	0.33	55	7.1
C77	14	64	11.8	387	5.0	1.15	0.26	59	5.1
C78	15.3	63	19.3	141	4.4	0.56	0.33	41	7.4
Ave.....	14.3	70	13.7	300	4.7	0.82	0.31	55	6.5
15% fat protein-deficient diet									
C79	17.5	78	13.1	221	9.0	1.20	0.24	32	5.4
C80	17	83	26.6	437	29.5	6.45	0.18	39	0.6
C81	22.3	38	13.8	143	7.6	0.87	0.36	41	4.7
Ave.....	19	66	17.8	367	15.4	2.84	0.26	37	3.6
High fat (33%) protein-deficient diet									
Ave.....	14.3	46	25.0	266	16.2	2.49	0.27	38	2.2

* Figures after decimal points indicate number of days.

† Phosphatase in Bodansky units per 100 cc. serum.

‡ Per lb. body weight.

shown in figure 2. The data on the high-fat (33 per cent) protein diet quoted from previous studies (1) are included in the table and graph for comparison.

The protein-depleted dogs survived longer on a low-fat diet than on a high-fat one. Diarrhea occurred frequently. Appetite generally remained good for 8

to 9 weeks and there was some food left thereafter. The dogs on 15 per cent fat diet had a better appetite and less diarrhea than the groups fed less fat. In spite of anorexia and diarrhea, the groups fed 2, 7 and 15 per cent fat all survived longer than those on the high-fat diet. Some of these animals lived as long as 21 or 22 weeks, while none of the dogs fed the high-fat (33 per cent) protein-deficient diet were alive after 18 weeks. Some of the most striking duodenal ulcers obtained were in the protein-deficient dogs on a low-fat diet; this finding will be considered elsewhere (9).

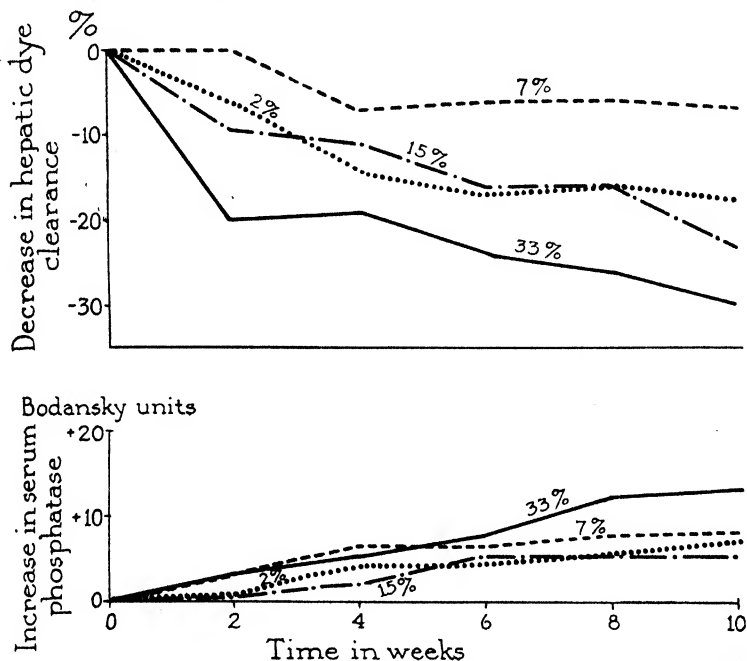


Fig. 2. Effect of exogenous fat (percentage indicates the fat content of the protein deficient diet).

There was some depression in the rate of Rose Bengal clearance, but this happened much later and was much less marked than for the high fat group. All three groups had some elevation of serum phosphatase. On an average, the 15 per cent fat group had higher maximum figures than the other two groups but not so high as the animals on the high-fat protein-deficient diet.

Hypercholesterolemia occurred even in the dogs fed the diet containing 2 per cent fat. The maximum increases above their respective initial values were 117 to 218, average 158 mgm. per cent. It was 103 to 218, average 185 per cent in the 7 per cent fat group. That of the 15 per cent fat group was 189 to 556, average 348 mgm. per cent.

The three dogs fed the 2 per cent fat protein-deficient diet and sacrificed at the end of the twelfth week all had very large livers. The average size was 22.0 grams per pound body weight as compared to 14.1 grams in the animals fed the high-fat protein-deficient diet and to 10.9 grams in the high fat control group which received 2 grams casein per pound body weight daily. Because of the low concentration of lipids (2.4 to 4.0, average 3.2 per cent), the total amount of liver lipids was not more than the high-fat control group nor was the amount per pound body weight increased. These livers were very friable, and water tended to separate out from the cytoplasm when the livers were ground for fat analysis.

The size of the livers of the other three animals on the same diet (survival period 15 to 21 weeks) was not larger than normal. One of them had a slightly higher concentration (8.5 per cent) of lipids than was found in the high-fat controls, while the other two were both within the control range.

The dogs on 7 per cent fat had normal sized livers with a normal concentration of liver lipids, as compared to the high-fat controls.

The protein-deficient diet with 15 per cent fat caused fatty infiltration of the liver. Two of the livers were only slightly higher (7.6 per cent and 9.0 per cent) than the control, and the third had 29.5 per cent of lipids. This particular liver was also slightly enlarged. The degree of fatty infiltration seems to increase with the increase of fat in the diet.

DISCUSSION. The effect of added bile salts on hepatic function and composition of the liver are best explained as due to an effect upon fat absorption. The deposition of lipids in the liver started soon after the animals were put on the deficient diet. This is at least true of the animals fed cholesterol (1). The early results on dye clearance indicate that bile salts intensify the initial effect of a high-fat protein-deficient diet on the liver. If the production of bile salts was reduced due to damage to the liver caused by protein-deficiency or cholesterol feeding, the bile salts added to the ration would tend to maintain fat absorption at a relatively high level. The effect of added bile salts suggests that a decreased flow of bile into the gut occurs soon after dogs are placed on a high-fat protein-deficient diet. Bile fistula dogs refuse to eat diets similar to those used in this study, so it has not been possible to study the bile directly. The effect of bile salts on fat absorption in protein-deficient animals could be studied by stool analysis, although such experiments on dogs are not very satisfactory. Member, Burger and Oppenheim (10) showed that cholic and glycocholic acid increased the absorption of cholesterol in rabbits as evidenced by the elevation of its content in whole blood and in the aorta. Our results indicated that bile salts increased absorption of fat and cholesterol in dogs as evidenced by the elevation of total lipids and cholesterol in the liver. However, serum total fatty acids and cholesterol had a tendency towards the lower limit of those without bile salts.

An increase of total serum fatty acids and cholesterol occurred when the protein-deficient diet contained only 2 per cent fat. That the degree of lipemia was related to fat intake is evidenced by the difference in serum cholesterol level on diets of different fat content. Some of the extra lipids in blood may have been mobilized from fat depots or synthesized from carbohydrate.

A high fat content in the diet may cause a higher elevation of serum phosphatase but the results were not consistent enough to permit any definite conclusion. A decrease in exogenous fat decreases the rate of fat accumulation in the liver and also the rate of depression of hepatic dye clearance. The results on serum phosphatase and dye clearance both indicate definitely that exogenous fat contributes materially to the impairment of liver function of the protein-deficient dog.

The effect of dietary fat on the liver of protein-deficient dogs is clearly demonstrated in this experiment. There was a gradation in the concentration of liver lipids from the dogs on the 2 per cent and 7 per cent fat protein-deficient diets up to those on 33 per cent although there were also a few exceptions. Though dogs on a low fat diet might have built up fatty acids from non-lipid materials, yet this synthesis seems to be limited, or at least not so marked as to cause excessive deposition in the liver. It is of interest to know whether or not a dog can be fattened like hogs or ducks, on a carbohydrate-rich diet. However, from the present study it seems improbable that the liver of dogs can be made highly fatty by a protein-deficient diet containing less than 15 per cent of fat. In this respect the effect of a low-fat or fat-free diet on dogs is probably different from that on rats (4) or on mice (5).

This study of the effect of dietary fat was complicated by the early appearance of anorexia and the frequent occurrence of diarrhea. There seemed to be little difference whether sucrose or dextrin was used as the source of carbohydrate. Three dogs on 2 per cent fat diet were sacrificed at the end of the twelfth week before their condition had been much influenced by diarrhea and inanition. Their liver lipid per pound body weight (0.64, 0.67 and 0.74 gram) was comparable to those of three other animals that were sacrificed when moribund (0.54, 0.87 and 1.24 grams).

SUMMARY

1. The fat which accumulates in the liver of a protein-deficient dog is largely exogenous in origin. The evidence for this statement is as follows:

a. Livers were uniformly higher in fat on the high-fat protein-deficient diet with or without cholesterol when bile salts were added to the ration.

b. The initial depression of hepatic dye clearance was more striking in the dogs with added cholesterol and bile salt than those fed only the cholesterol supplement.

c. The fat content of liver increased as the fat content of the protein-deficient diet was increased from 2 to 7 to 15 and to 33 per cent.

2. The increase of Rose Bengal clearance comes later and is less marked in the dogs on the low-fat diet.

3. Some increase of total serum fatty acids and cholesterol occurred in protein-deficient dogs on a diet poor in fat.

4. The reduction of hepatic dye clearance that occurs during the first 1 to 2 weeks when a dog is fed a protein-deficient diet with cholesterol is largely due to an accumulation of lipids in the liver.

5. Exogenous fat impairs liver function in the protein-deficient dog.

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THE RELATIVE SENSITIVITY OF THE MUCOSAL AND PERITONEAL SURFACES OF GUINEA-PIG ILEUM TO HISTAMINE, ACETYLCHOLINE AND SPECIFIC ANTIGENS¹

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During the investigation by Nicoll and Campbell (1, 2) of *in vitro* anaphylaxis, it was discovered that suitable antigens caused no response of the intestinal strips either when the strips were everted or when antigen was injected into the lumen of the strip. It was apparent, therefore, that in order to set off the mechanism responsible for anaphylactic contraction of intestinal smooth muscle the antigen must come into contact with the peritoneal surface.

These earlier observations have been repeated and extended to include studies dealing with the response to histamine and acetylcholine as well as to antigens of both relatively small and large molecular size.

Preparations of intestinal strips. The studies were made entirely with guinea-pig intestine. No particular selection was made as to sex or to the weights of the animals, which varied from 300 to 500 grams. The animals were usually bled to death by cardiac puncture, since the tissues then seemed to react more normally than when the animal was killed by a blow at the base of the skull. This latter method, which was used in most of the earlier studies, often produced a "refractoriness" in intestinal strips which disappeared only after prolonged exposure to aerated Tyrode's solution or storage in the cold. The abdomen was opened as soon as possible and the lower third of the small intestine was removed and placed in warm balanced salt solution.² The large strip was then cut into smaller sections of about 15 cm. to facilitate manipulation and was carefully flushed with warm salt solution. Portions not to be used immediately were covered with salt solution and stored in the refrigerator. Strips for immediate testing were then cut into 25-30 mm. lengths and the ends were tied tightly with silk thread. They were then suspended on a glass support and placed in the 100-ml. muscle bath which has been described by Campbell and McCasland (3).

The following method was used to evert muscle strips: a thin glass rod with a small knob at one end was passed into the intestinal lumen until the bulb emerged. A ligature was then made near the bulb and the free end was pulled over and past the knob until complete eversion was achieved, the entire procedure

¹ This investigation was financially supported by a Rockefeller Foundation fund for immunochemistry research.

² The balanced salt solution used was a slight modification of Tyrode's formula and contained per 100 ml. of solution: $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 0.0213 gram, KCl 0.0195 gram, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.0193 gram, NaHCO_3 0.1015 gram, NaCl 0.800 gram, and glucose 0.1000 gram. The final pH at 37°C. was 8.2.

being carried out in a petri dish containing warm salt solution. A suitable portion was then ligated at both ends and placed in the muscle bath for testing. The records clearly show that the failure of such everted preparations to react was not due to the above manipulation, since re-everted strips showed only a slight loss in irritability.

Studies on *in vitro* anaphylaxis were performed on guinea pigs which had been sensitized either to arsanilic-azo-ovalbumin or to histamine-azo-ovalbumin. The former was given intra-abdominally in four injections of 15 mgm. each at 48-hour

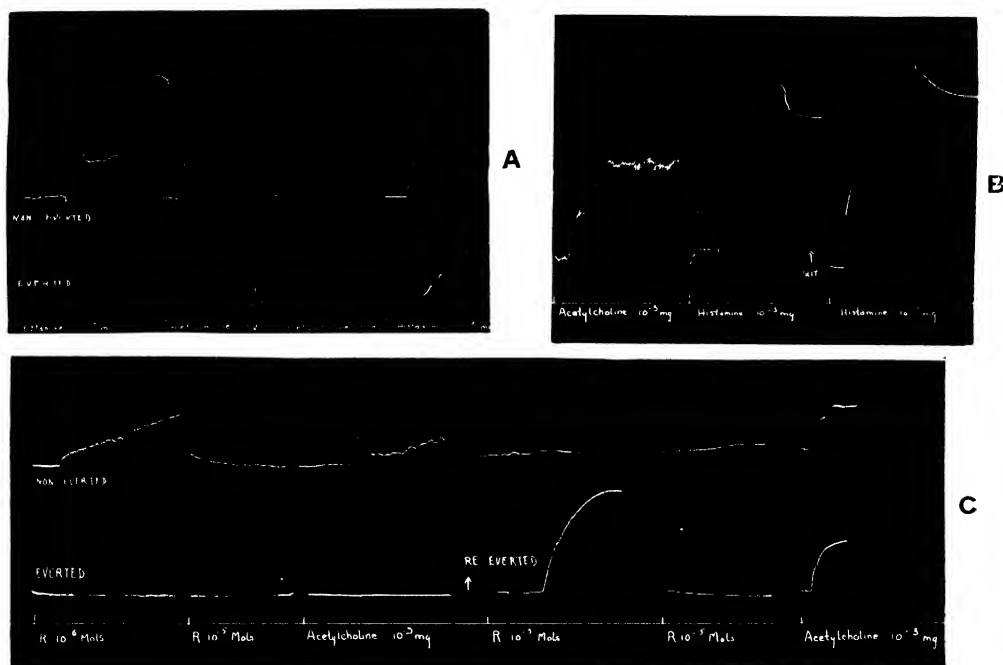


Fig. 1. Kymograph records showing the effects of specific and non-specific chemical agents on everted and non-everted strips of guinea pig ileum.

intervals and the latter in two injections of 25 mgm. each. The animals were used 14 or more days following the final injection.

RESULTS. Typical effects obtained from normal and everted intestinal strips are given in the kymograph records of figures 1 and 2.

Figure 1A. Everted and non-everted strips were first exposed to 10^{-3} mgm. of histamine and then 1 mgm. of acetylcholine in the 100-ml. bath. The non-everted strip responded normally, while the everted preparation showed no response. After a longitudinal slit was made, the everted as well as the non-everted responded to both acetylcholine and histamine.

Figure 1B. This is an additional record to show that a normal non-everted strip apparently reacts normally after being subjected to a longitudinal incision.

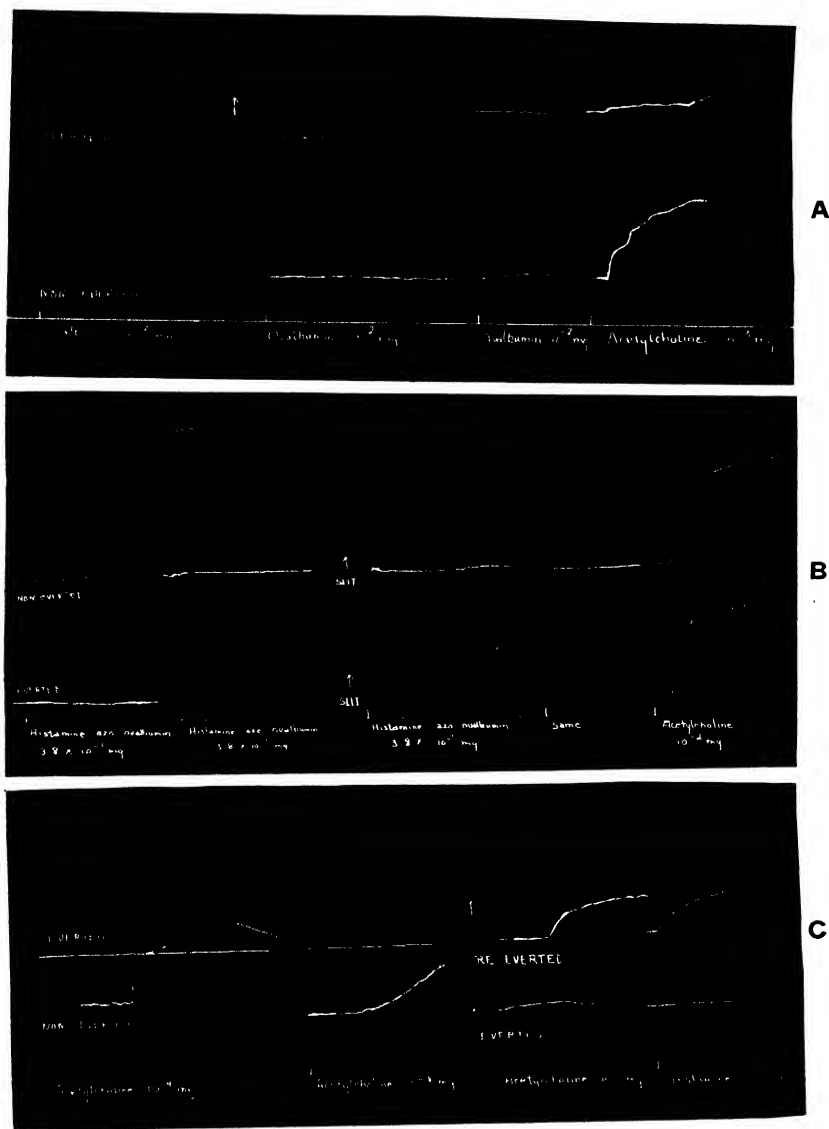
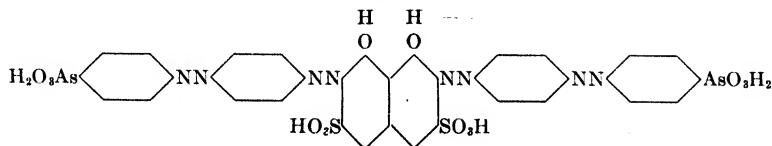


Fig. 2. Kymograph records showing the effects of specific and non-specific chemical agents on everted and non-everted strips of guinea pig ileum.

Figure 1C. These strips were taken from a guinea pig which had been sensitized to arsanilic-azo-ovalbumin. The test antigen was



The addition of 10^{-6} moles of this material produced no responses in the everted strip, but the non-everted strip gave a normal anaphylactic response. After the tissue was washed and allowed to return to its normal state, a second addition of antigen caused no response, indicating that desensitization had taken place. The addition of acetylcholine caused the non-everted strip to contract, proving its irritability. The everted strip was then re-everted, thus exposing the peritoneal surface. When antigen was again added, the re-everted strip gave a typical anaphylactic response and was desensitized, as evidenced by its failure to respond to a second exposure of antigen. Both strips subsequently responded to acetylcholine, proving their viability.

Figure 2A. Intestinal strips from guinea pigs sensitized to histamine-azo-ovalbumin were tested against ovalbumin.³ Here again the everted strip failed to respond to antigen until re-everted.

Figure 2B. The same type of strips was used here as those of 2A. Histamine-azo-ovalbumin was used as the shocking antigen. Here again the everted strip did not contract until it was slit longitudinally, thus allowing the antigen to come into contact with the peritoneal surface of the intestine.

Figure 2C. Everted and non-everted strips were first tested with acetylcholine. The former contracted, while the latter showed no activity. The strips were then reversed and the initially non-everted muscle was everted while the initially everted strip was re-everted. Responses were then clearly reversed and the initially inactive intestine became responsive upon re-eversion.

DISCUSSION. The foregoing data demonstrate that the smooth muscle of isolated intestinal strips will respond to specific (antigens) or to non-specific (histamine, acetylcholine) chemical stimuli only when the peritoneal surface of the gut comes in contact with these agents. Apparently such chemical substances cannot diffuse through the relatively thick mucosal layer and hence are prevented from reaching the sites responsible for muscle activation. However, it is of interest to note that the muscle response to both large and small molecular substances is extremely rapid when applied to the peritoneal surface, although this surface consists of a serous membrane of appreciable thickness. In fact, the response is so rapid that it would appear as though actual penetration to the muscle layer were not necessary.

King and Robinson (4) reported contraction of *muscularis mucosae* in isolated mucosal strips upon exposure to acetylcholine or histamine. Since we obtained no contraction of everted strips, it must be assumed that either their recording system was more sensitive than ours or that activation was induced by stimulation from the inner mucosal surface rather than from the epithelial surface.

The failure of such stimulants to induce contraction of intestinal muscle when administered orally is due to absorption or detoxification in the mucosal tissue or portal circulation. Of the simple substances, atropine is an exception, but it apparently reaches the intestinal muscles by way of the circulatory system and not by direct diffusion. It is well known that antigenic materials such as ovalbumin can pass through the intestinal mucosa of normal animals. However, it would appear from the foregoing results that the antigen must reach the intesti-

³ The sensitizing antigen was prepared by coupling the diazonium salt of histamine to ovalbumin. Enough of the original ovalbumin specificity was retained to produce corresponding hypersensitivity. It was of interest to note that there was no evidence of a refractoriness to histamine, which might have been expected.

nal muscle via the circulation if reactions are obtained following ingestion of antigen in a sensitized animal.

SUMMARY

Surviving intestinal strips have been shown to react to specific and non-specific substances only when these agents have access to the peritoneal surface of the gut. The possible reasons for this effect have been discussed.

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COMPENSATORY GROWTH OF THE KIDNEY AFTER UNILATERAL NEPHRECTOMY IN THYROIDECTOMIZED RATS

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Thyroidectomy in rats is known to result in pronounced structural changes in the pituitary, consisting of increase in size and weight, reduction in numbers of acidophils, and increase in numbers of basophils, many of which are distended with hyaline material (thyroidectomy cells). Such a rearrangement in the cellular pattern of the pituitary might cause alteration in the relative proportions of the various pituitary hormones. The correlation of alterations in the secretory products with histological changes in the pituitary might give clues as to which pituitary cells produce the various hormones.

In the present experiments, the interest has been centered on whether, after one kidney is removed, there would result compensatory increase in growth of the remaining kidney, under the conditions of decreased numbers of acidophils in the pituitary following thyroidectomy. The growth hormone is commonly ascribed to acidophil secretion, and possibly compensatory hypertrophy of the kidney is dependent upon the acidophils.

In previous experiments (10) the abundance of thyrotropic factor in the pituitary of dwarfed thyroidectomized rats was taken to indicate that this factor was probably not produced by the acidophils, so greatly reduced by thyroidectomy, but was probably produced by basophils transformed into vacuolated thyroidectomy cells. Any change in gonadotropin content of the pituitary of thyroidectomized animals is one of decrease and therefore is probably not a product of the many thyroidectomy cells, but is probably a product of a different basophil than the thyroidectomy cells. Assays of adrenotropic hormone content of the rat's pituitary are not feasible, so indirect evidence was obtained by studies of adrenal weights (12), and of compensatory adrenal hypertrophy after removal of one adrenal in rats after combined thyroidectomy and castration (13) which suggested that the adrenotropic factor was produced not by acidophils but by a basophil different from the thyroidectomy cells and castration cells. Castration causes increase in numbers of basophils (castration cells) which secrete excess gonadotropic hormone. These castration cells are different structurally and functionally from thyroidectomy cells (11). After castration there is no loss of acidophils, which are commonly regarded as producing the growth hormone, and MacKay (5) found that castration did not affect compensatory renal hypertrophy after unilateral nephrectomy.

In the present experiments, preliminary data having been presented in abstract previously (14), the same general problem of trying to assign hormones to specific

¹ Aided in part by a grant from the Faculty Research Committee of the University of Pennsylvania.

types of pituitary cells was extended to the study of kidney growth in thyroidectomized rats. Since assay of the growth factor of the pituitary of rats is not possible, the experiments were planned to obtain indirect evidence of visceral growth by studying the compensatory renal enlargement after unilateral nephrectomy, in order to see if the structural changes in the pituitary after thyroidectomy interfered with kidney growth. That the pituitary is necessary for compensatory renal hypertrophy has been shown by McQueen-Williams and Thompson (6) in rats, and by Winternitz and Waters (8) in dogs. In a previous publication by the present author (9) it had been suggested that the retardation of skeletal and visceral growth resulting from thyroidectomy might be ascribed to decreased

TABLE 1

Compensatory renal hypertrophy after removal of one kidney in thyroidectomized rats compared with that in rats with thyroids intact. Averages

	YOUNG MALES		ADULT FEMALES	
	With thyroids intact	With thyroids removed	With thyroids intact	With thyroids removed
Body weights, controls, kidneys intact. Mean of 12 rats (gm.).....	251 \pm 15*	239 \pm 13	228 \pm 10	224 \pm 10
Unilaterally nephrectomized. Mean of 12 rats (gm.).....	239 \pm 14	225 \pm 15	230 \pm 9	226 \pm 10
A = $\frac{1}{2}$ wt. of both kidneys of controls. Mean of 12 rats (gm.).....	1.108 \pm 0.067	0.930 \pm 0.053	1.020 \pm 0.044	0.861 \pm 0.044
B = wt. of remaining kidney after first is removed. Mean of 12 rats (gm.).....	1.611 \pm 0.087	1.318 \pm 0.054	1.284 \pm 0.060	1.140 \pm 0.075
Compensatory hypertrophy, $\frac{B-A}{A} \times 100 = \%$. Mean of 12 pairs.....	46.7 \pm 3.5	43.6 \pm 4.6	27.0 \pm 5.5	30.1 \pm 7.2
No. days after thyroidectomy.		32-123		49-125
No. days after nephrectomy...	21-88	21-88	21-81	21-81

* Standard error.

secretion of growth hormone by a pituitary greatly reduced in acidophils. It was thought that the present experiments might give evidence for or against such a hypothesis.

METHODS. In one series of experiments, four litter-mate young male rats made up each experimental group, and consisted of 1, control intact rat; 2, rat in which one kidney had been removed; 3, thyroidectomized rat; 4, thyroidectomized rat in which one kidney had been removed. Sufficient time after thyroidectomy was allowed for appearance of histological changes in the pituitary, after which one kidney was removed. In all but one group of rats one adrenal and/or one testis was also removed from the nephrectomized rats so as to place upon the pituitary an added burden of producing hormones known to be neces-

sary for the compensatory enlargement of the remaining adrenal and testis. When there were more than 4 male rats in the same litter, an extra rat was used in the same group. In such case the weights of the 2 rats subjected to the same operation were averaged and used as a single value.

At intervals after nephrectomy, varying from 21 to 88 days, the rats were killed and their organs weighed. Addis and Lew (1) showed that compensatory hypertrophy of the kidney was usually completed by about 20 days. Suitable checks were made of the completeness of the thyroidectomy. As diet is known to affect the rate of kidney hypertrophy, all rats were kept on a constant diet of Purina and greens.

A second series of experiments was carried out with adult female rats, in exactly the same manner except that these rats were not litter-mates but had been used for breeding and were selected in groups of four on the basis of comparable body weights.

RESULTS. Compensatory hypertrophy was calculated as the percentage difference between the weight of the remaining kidney after unilateral nephrectomy and one half the weight of both kidneys of the control. It will be seen from the table that the weights of the kidneys in the thyroidectomized rats with both kidneys intact are considerably less than in the rats with thyroids intact, in the case of the adult females as well as in the growing males. In spite of this, the compensatory hypertrophy in thyroidectomized rats was very similar to that in rats with thyroids intact, as may be seen in the table. In other words, thyroidectomy reduced the bulk of kidney tissue, but did not reduce the capacity of the remaining tissue to compensate for loss of one kidney.

DISCUSSION. Since the present experiments were begun a paper has been published by McQueen-Williams and Thompson (6) comparing compensatory hypertrophy of the kidney in thyroidectomized, hypophysectomized and normal rats. They found that thyroidectomy did not interfere with renal hypertrophy. They estimated hypertrophy by taking the weight of the surgically removed kidney and comparing it with the weight of the remaining kidney from 10 to 86 days later. This method would seem to give an exaggerated index of growth because of the increase in weight which would naturally occur in 86 days in growing rats independent of any extra stimulus to growth from unilateral nephrectomy, except in so far as this is corrected for by expressing values as ratio of kidney weight at operation to body weight at operation and comparing this with ratio of kidney weight at death with body weight at death. They stated that a group of normal rats served as "further control weights of kidneys." They were not concerned with relating this to histological changes in the pituitary. The results presented in the present paper are therefore a corroboration of their results obtained by a somewhat different experimental procedure.

The previously expressed idea (9) that retardation in kidney growth after thyroidectomy might be ascribed to acidophil loss must be modified now in the light of the present experiments, since in thyroidectomized rats with reduced acidophils, hypertrophy of the remaining kidney is readily accomplished. Furthermore, data in the literatures subsequent to the expression of that idea indicate

that with deficient thyroid, the administration of thyroid extract restores the pituitary histologically and restores body growth, while the administration of anterior pituitary growth hormone alone does not cause restoration (Shelton, Tager and Hoyt, 7; Beard, 3; Evans, Simpson and Pencharz, 4).

That the acidophil is related to kidney growth and function is indicated by the experiments of Barnett, Perley and Heinbecker (2) who found that patients with acromegaly (acidophil tumor) had urea clearance above normal and 2 patients with Cushing's syndrome had renal function within normal limits. They interpreted this to mean that the "humoral agent affecting kidney function is secreted by the eosinophil cells."

Since acidophil cells are commonly regarded as the source of growth hormone, it was a surprise to find compensatory renal enlargement occurring adequately under the conditions of acidophil loss incident to thyroidectomy. It seems necessary to conclude that kidney growth is either not dependent upon acidophils or that it is not necessarily proportional to the number of acidophils. Perhaps there is a great margin of safety and reserve capacity in the pituitary, just as there is an abundance of excess liver tissue.

SUMMARY

Although thyroidectomy retards the growth of the kidneys, it does not prevent compensatory hypertrophy of one kidney after removal of the other.

Decrease in numbers of acidophil cells in the pituitary incident to thyroidectomy does not prevent compensatory enlargement of the kidney; consequently kidney growth is not proportional to the number of acidophils present.

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BLOOD OXYGEN SATURATIONS AND DURATION OF CONSCIOUSNESS IN ANOXIA AT HIGH ALTITUDES

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Survival times at high altitudes following failure of oxygen equipment in military aircraft has been a critical problem. With the advent of pressurized cabins for stratospheric flying in both military and commercial aviation, the problem of survival in the event of mechanical failure resulting in loss of cabin pressure becomes an important consideration. Two investigators, Mackenzie (1) and Hemingway (2), have reported on the duration of consciousness of human subjects breathing air at high altitudes. Only one, Hemingway (2), determined blood oxygen saturations during the experiments. In each instance the studies were made with a number of individuals in the low pressure chamber. Both investigators used the ability to write as a measure of useful consciousness. It is the opinion of the authors of this paper that the duration of consciousness which could be considered useful is shorter than that measured by handwriting ability.

The purpose of this investigation is to present the following information on the reactions of man to breathing air at altitudes ranging from 28,000 to 38,000 feet: *a*, period of useful consciousness; *b*, times to tremor and imminent unconsciousness; *c*, blood oxygen saturations at termination of useful consciousness, at tremor, and at imminent unconsciousness.

METHODS. Subjects consisted of flight students and Hospital Corpsmen ranging in age from 18 to 30 years. The 6 Hospital Corpsmen were used as subjects at each of the 4 altitudes, but each flight student acted as subject only once.

Blood oxygen saturations were estimated with a Millikan oximeter by conventional oximeter technique. Pressure altitudes were determined by a mercury manometer, and oxygen pressures in the chamber were recorded from a continuous oxygen analyzer. The accuracy of the oxygen analyzer was checked periodically against the Haldane-Henderson gas analyzer.

At sea level the oximeter was standardized and the subject was fitted with an oximeter ear piece. Initial blood oxygen saturation, with the subject breathing air at sea level, was set at 98 per cent. Using diluter-demand oxygen equipment the subjects were taken one at a time to a simulated altitude in the low pressure chamber. To insure that the subject was receiving 100 per cent oxygen prior to mask removal, the oxygen regulator was set to the "100 per cent oxygen" position at 27,000 feet during ascent on each run. Maximum ventilation was maintained in the chambers while at altitude, and, by means of a suction pump, samples of the chamber air were constantly drawn from just above the subject's head

¹ This problem was suggested and the preliminary phases of the investigation were supervised by Lt. C. S. White (MC) USN.

through the oxygen analyzer. After approximately 5 minutes at altitude, the instruments were read, and the subject had his mask removed and breathed ambient air. He was given the task of naming and sorting cards from an ordinary pack, placing cards in one of four slots in a box according to card suit.

Two observers accompanied the subject on each run. It was the task of one observer to remove the mask and to report the subject's reactions, while the other recorded data and read the instruments. Both observers were placed in the chamber so that their exhaled oxygen was always carried away from the subject. Times to error, onset of convulsive-like movements, and mask replacement

TABLE 1

The mean and the range of times to error, tremor and mask replacement on 25 subjects

ALTITUDE	TIME IN SECONDS					
	Error		Tremor		Mask replacement	
	Mean	Range	Mean	Range	Mean	Range
<i>feet</i>						
28,000	110 \pm 5.5	50-178	106 \pm 4.2	70-160	141 \pm 5.3	100-210
30,000	73 \pm 3.1	40-100	71 \pm 2.2	45-100	98 \pm 2.2	80-115
35,000	46 \pm 2.2	25-68	51 \pm 1.6	40-70	72 \pm 1.6	60-90
38,000	35 \pm 1.3	20-45	35 \pm 1.3	25-45	47 \pm 1.4	35-60

TABLE 2

The mean and the range of blood oxygen saturations at the time of error, tremor and mask replacement on 25 subjects

ALTITUDE	SATURATION IN PER CENT					
	Error		Tremor		Mask replacement	
	Mean	Range	Mean	Range	Mean	Range
<i>feet</i>						
28,000	61 \pm 1.77	45-76	62 \pm 1.71	45-78	54 \pm 1.85	40-73
30,000	63 \pm 1.6	48-78	63 \pm 1.8	45-82	54 \pm 1.6	40-68
35,000	70 \pm 1.6	50-84	66 \pm 1.7	48-82	58 \pm 1.6	40-72
38,000	63 \pm 1.6	45-82	64 \pm 1.9	45-80	57 \pm 1.4	40-66

were noted and recorded. Readings of the oximeter and oxygen analyzer were recorded at 10 second intervals. The mask was replaced after the subject had lost physical and mental co-ordination, and unconsciousness was imminent. Readings were continued until the blood oxygen saturation had returned to approximately its original value after which descent was begun.

RESULTS. Table 1 presents the mean and the range of times to error, tremor, and mask replacement on 25 subjects at each of the 4 altitudes studied.

Table 2 gives the average blood oxygen saturations at the appearance of error, tremor, and imminent unconsciousness. In figures 1 to 4 the fall in blood oxygen saturations, following the change from 100 per cent oxygen to air, is graphically illustrated for each of the 4 altitudes. These figures show mean and individual

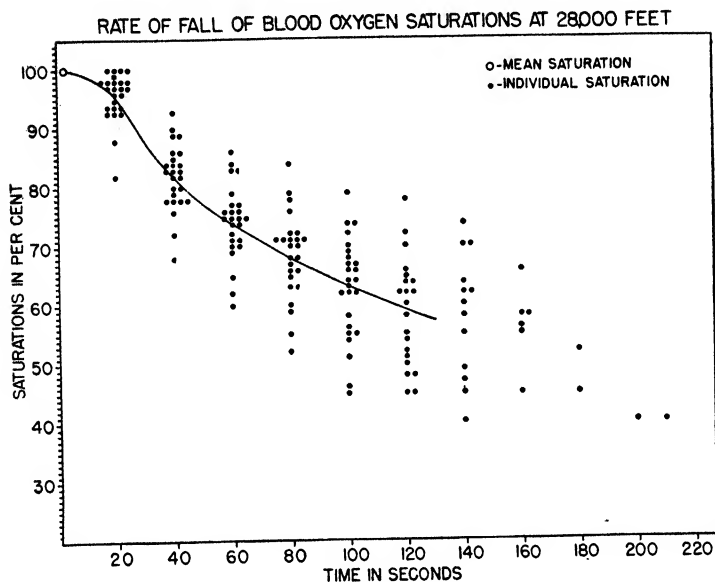


Fig. 1. Mean and individual blood oxygen saturation at 20 second intervals following mask removal at 28,000 feet. Saturations are included for each individual until the time the mask was replaced. Initial blood oxygen saturations were 100 per cent in all cases.

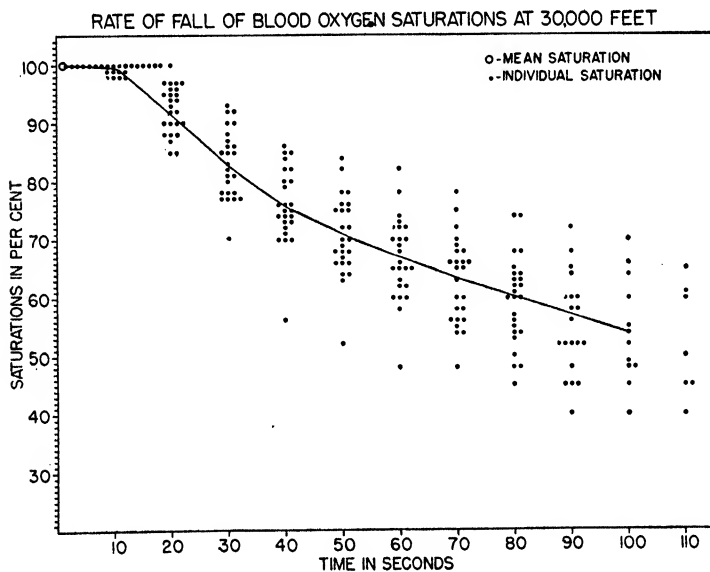


Fig. 2. Mean and individual blood oxygen saturations at 10 second intervals following mask removal at 30,000 feet. Saturations are included for each individual until the mask was replaced.

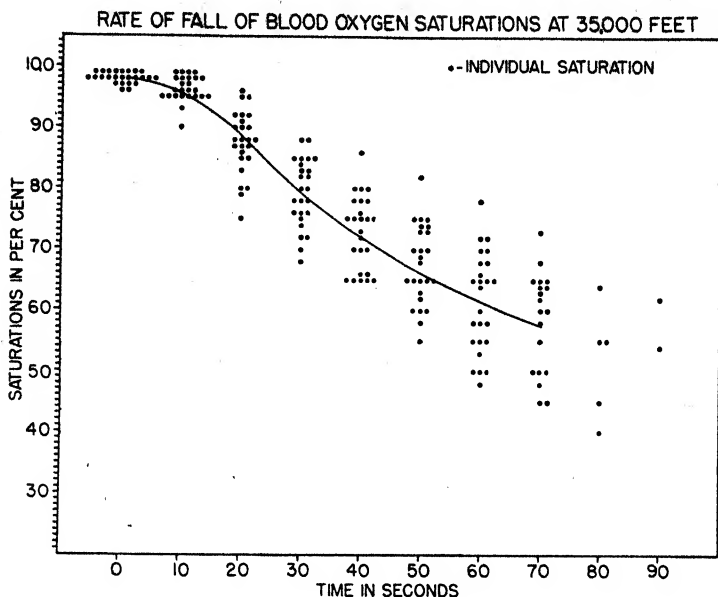


Fig. 3. Mean and individual blood oxygen saturations at 10 second intervals following mask removal at 35,000 feet. Saturations are included for each individual until the mask was replaced.

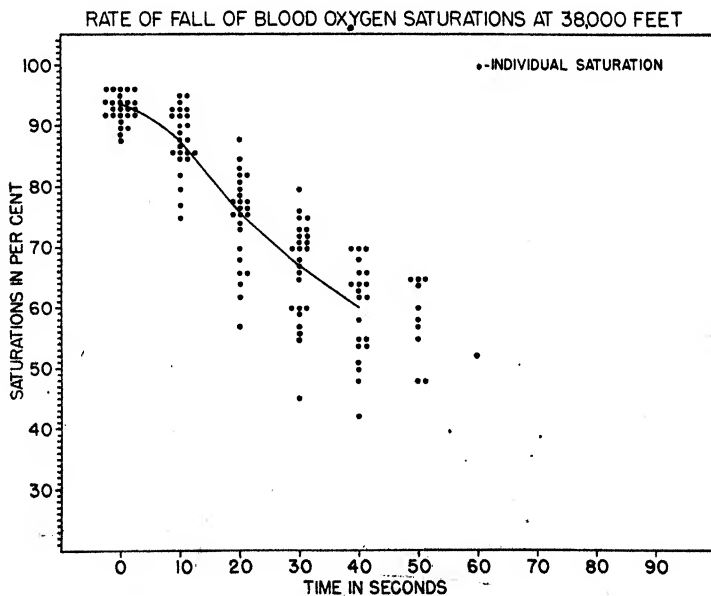


Fig. 4. Mean and individual blood oxygen saturations at 10 second intervals following mask removal at 38,000 feet. Saturations are included for each individual until the mask was replaced.

saturations at 10 second intervals for altitudes 30,000, 35,000 and 38,000 feet; for 28,000 feet they are presented for every 20 seconds. Comparison of the average rates of fall of blood oxygen saturations at the 4 altitudes is shown in figure 5.

Discussion. Following mask removals at high altitudes most individuals pass through two definite phases. The first stage can be considered as useful consciousness and may be defined as that period up to the time the subject first begins to make errors. The second is that phase in which the subject is conscious, but is so mentally confused that he either continues to make mistakes or just sits with no apparent awareness of his situation. Soon after the latter phase is reached, unconsciousness is imminent, and unless the subject is supplied with oxygen he will collapse. Most individuals lose control of muscular co-ordination at about the time that errors are made.

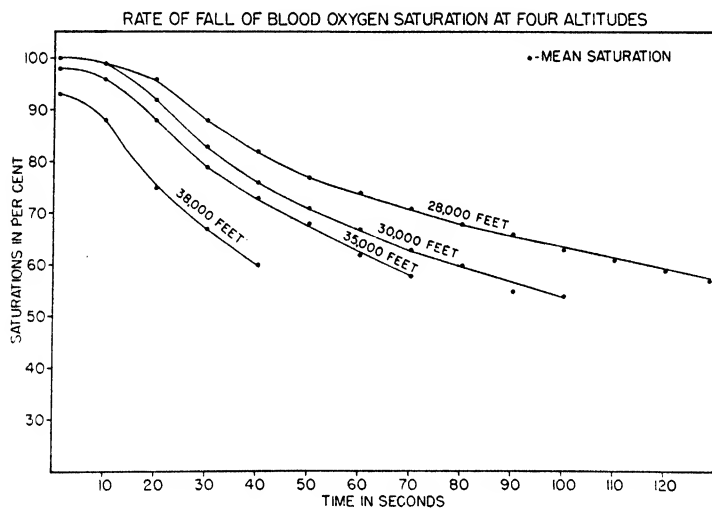


Fig. 5. A comparison of the fall of average blood oxygen saturations following mask removals at four altitudes.

In preliminary investigations it was found that if more than three people remained at altitudes in a low pressure chamber for a few minutes, it was impossible to retain the desired ambient oxygen pressure even though maximum ventilation was maintained in the chamber. This increase in oxygen pressure was caused by an accumulation of exhaled oxygen from the individuals wearing masks. Since the difference in oxygen tension in ambient air between 28,000 and 30,000 feet is only 4.4 mm. Hg, an increase of 1 mm. Hg is equivalent to an increase of 10 seconds in duration of consciousness at this range of altitudes. This is in agreement with conclusions of Mackenzie (1), that, "for each 1,000 feet increase in altitude between 26,000 and 32,000 feet, the duration of useful consciousness decreased by approximately twenty seconds." At higher altitudes, errors from this source become less acute, but the importance of maintaining the exact am-

bient oxygen pressure while tests of this kind are being conducted is evident if results are to be reliable.

In an attempt to find some means of measuring useful consciousness, card naming and sorting was selected because each new card demands a new verbal response and the selection of one of four slots for placement of the card. With this procedure it was possible to establish the time when errors and mental confusion first occurred. It seems reasonable that if an individual is unable to perform this simple task of card sorting at altitude in a low pressure chamber, he certainly would be unable to carry out the more complicated duties required of him in aircraft. Experience with handwriting as a criterion of useful consciousness-

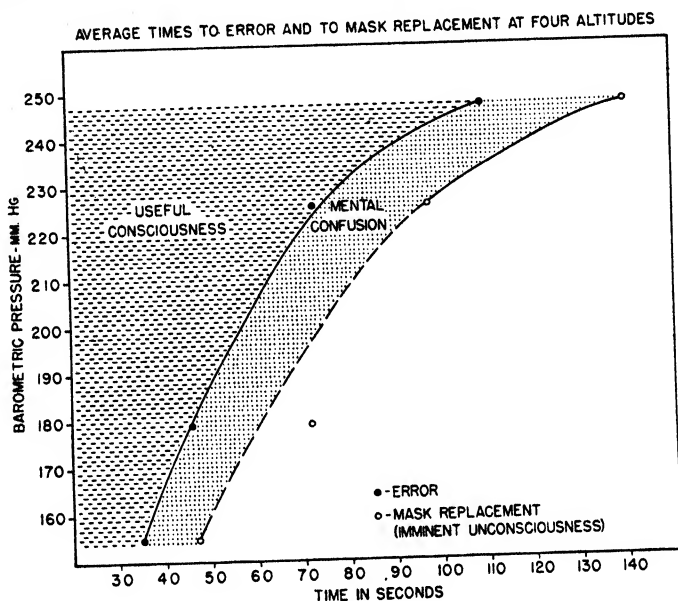


Fig. 6. The period of useful consciousness in relation to mask replacement time (imminent unconsciousness) at 28,000, 30,000, 35,000 and 38,000 feet.

ness has indicated that many persons can continue writing legibly after mental confusion has set in. In these instances, the subject, after his mask had been replaced, often stated that he had no remembrance of having written the last few lines. This probably accounts, in part, for the fact that the average times of useful consciousness presented here are shorter than those found by investigators who have used handwriting as the measure of useful consciousness.

Figure 6 illustrates graphically the period of useful consciousness in relation to mask replacement time at the four altitudes studied. With the exception of 35,000 feet, the period of useful consciousness is approximately three-fourths of the time to mask replacement. At 35,000 feet the time to error was 64 per cent of the total time. A study of figure 6 shows that the time to error at 35,000 feet

follows the trend of the curve when compared with the other altitudes, but the time to imminent unconsciousness is relatively longer. No immediate explanation for this difference is apparent, since the subjects, with the exception of the six corpsmen who acted as subjects at all four altitudes, were selected at random from flight students.

The time of useful consciousness was reduced by two-thirds between 28,000 and 38,000 feet; it was shortened approximately one-third between 28,000 and 30,000 feet. The average reduction in time to error for each 1,000 feet for the range of altitudes studied, was 18 seconds between 28,000 and 30,000 feet, 5 seconds from 30,000 to 35,000 feet, and 4 seconds between 35,000 and 38,000 feet.

Mackenzie (1) reported the average time of useful consciousness at 28,000 feet to be 181 seconds with flying personnel and 154 seconds with nonflying personnel. It is difficult to account for the fact that the average of 141 seconds to imminent unconsciousness at 28,000 feet as found in this work is shorter than either average given for useful consciousness by Mackenzie, except on the basis of increased oxygen partial pressure with several people in the chamber. At 30,000 feet the same trend in comparison with data previously published is evident. At 35,000 feet the average of 72 seconds to imminent unconsciousness, as presented in this paper, agrees with Hemingway's (2) average of useful consciousness. This latter comparison emphasizes the difference in end point when the two measurements of useful consciousness are used, namely, card naming and handwriting. Armstrong's (3) times to coma of 90 seconds at 30,000 feet and 50 seconds at 38,000 feet agree favorably with the data here presented.

The six Hospital Corpsmen who acted as subjects at each of the four altitudes were experienced low pressure chamber technicians and no element of fear or anxiety entered into their performance at altitude. All other subjects were flight students with experience in aircraft but little or no previous experience in low pressure chambers. When the six Hospital Corpsmen are considered by themselves, they give averages at all altitudes that are consistently 1 to 9 seconds longer than those of the flight students.

In this discussion average times of useful consciousness have been emphasized, and it should be highly significant for aviation personnel to know these times for various altitudes. However, it is also important to realize that variations in tolerance among individuals is great, and that some persons begin to make errors in much shorter times than others.

It has been shown, (Houston, 4), that the rate of respiratory exchange plays an important part in determining the blood oxygen saturation at 16,000 feet with subjects breathing air. This factor of respiratory rate was not controlled or measured in the work reported here, with the exception that subjects could not hold their breath and call the cards as directed. However, it should be pointed out that this factor is also an uncontrolled variable following oxygen failure in aircraft.

In figures 1 to 4 the fall in blood oxygen saturations following the change from 100 per cent oxygen to air is graphically illustrated for each of the 4 altitudes. These graphs indicate an initial lag in the rate of loss of oxygen from the blood

which is followed by a sharp drop and then a constant rate of fall to the time of imminent unconsciousness. The initial lag in loss of blood oxygen persists longer at the lower altitudes. In the first minute following mask removals at 28,000 feet, blood oxygen saturation had decreased an average of 26 per cent; at 30,000 feet this decrease was 33 per cent; at 35,000 feet it was 36 per cent. At 38,000 feet, the highest altitude investigated, the saturation had dropped 33 per cent in 40 seconds, or at a rate of about 50 per cent per minute.

In this investigation it was found that the average blood oxygen saturation for the 100 exposures at the time when errors were made was 64 per cent. It has been previously indicated that the average times of tremor and error are identical; average blood oxygen saturation at tremor was 64 per cent. The saturation at imminent unconsciousness was 56 per cent.

SUMMARY AND CONCLUSIONS

1. Flight students and Hospital Corpsmen were taken singly to simulated altitudes of 28,000 feet and above in a low pressure chamber and had their masks removed. While at altitude, the subject performed a simple task of card sorting and his reactions were noted and recorded. These activities were continued until unconsciousness was imminent at which time the mask was replaced and 100 per cent oxygen was administered. Blood oxygen saturations were estimated throughout the procedure with a Millikan oximeter. Ambient oxygen pressure was determined with a continuous oxygen analyzer. This procedure was repeated for 25 individuals at each of four altitudes: 28,000, 30,000, 35,000 and 38,000 feet.

2. Times to imminent unconsciousness were 141 seconds at 28,000 feet, 98 seconds at 30,000 feet, 72 seconds at 35,000 feet and 47 seconds at 38,000 feet.

3. Average times of useful consciousness, as determined by the appearance of first error in card sorting, were 110 seconds at 28,000 feet, 73 seconds at 30,000 feet, 46 seconds at 35,000 feet and 35 seconds at 38,000 feet.

4. Times of useful consciousness at altitudes above 30,000 feet plot as a straight line curve, but below this altitude the curve swings toward the horizontal as it approaches the altitude at which individuals remain conscious indefinitely.

5. The period of useful consciousness was found to be approximately $\frac{3}{4}$ of the total time consciousness was retained.

6. Blood oxygen saturations averaged 64 per cent at the appearance of first error, and 56 per cent at imminent unconsciousness with very little variation between altitudes at the appearance of any particular anoxic symptom.

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THE EFFECTS OF FEEDING THYROID SUBSTANCE AND OF ADRENALECTOMY ON THE ACTIVITIES OF SUCCINOXIDASE AND CYTOCHROME OXIDASE IN THE LIVER TISSUE OF RATS¹

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After adrenalectomy there appears to be some decrease in the activity of cytochrome oxidase in rat liver (1). Since Schneider and Potter (2), using a more precise method for enzymatic assay than has hitherto been available, found that most tissues contain cytochrome oxidase in wide excess, this enzyme may not be limiting in most oxidative reactions. It has been shown that liver slices from adrenalectomized rats had a lower rate of oxygen consumption in the presence of sodium succinate than slices from normal animals (3). The succinoxidase system is composed of succinic acid dehydrogenase, cytochrome c and cytochrome oxidase, and occurs in most tissues where it takes part in the utilization of succinate as a metabolite and in the oxidation of pyruvate and acetate through the Krebs cycle (4). Since an assay method for this enzyme is available (2) with that of cytochrome oxidase we thought it important to extend our investigation to succinic acid dehydrogenase, an enzyme which may be more limiting in cell reactions than cytochrome oxidase.

It was thought that a more sensitive enzyme reaction might be developed by giving desiccated thyroid prior to the adrenalectomy. When the oxidation enzyme reactions of the cells are accelerated or the cells stimulated to produce more respiratory enzyme, as occurs when thyroid substance is fed, any modifying action exerted by the adrenal cortex might be expected to show up more clearly.

METHODS. Young males of the Long-Evans strain of hooded rats, weighing from 80 to 130 grams, were divided into three main experimental groups. Since the controls were treated somewhat differently according to the type of experimental treatment given, each experimental group had its own control group. The control groups were treated and fed exactly the same as the experimental rats except for the hormonal treatment.

The rats of group 1b were fed 400 mgm. of desiccated thyroid (Armour) per animal daily for fourteen days. The thyroid substance was mixed with powdered Rockland rat food. The rats of group 1b and their control group, 1a, were given all the food they wanted. The experimental rats of group 2 were adrenalectomized and given 1 per cent NaCl as drinking water until the 3rd day after operation. The average survival time after adrenalectomy in this strain was 11 days. Less than 1 per cent of our operated animals survived beyond the 15th day without treatment. The adrenalectomized rats were sacrificed for experiment on the 7th day after operation. Five adrenalectomized rats, group 2c,

¹ Aided by a grant from the Committee of Endocrinology of the National Research Council.

were given 0.5 ml. of Wilson's adrenal cortical extract per day for the 7-day period. The group 2a rats were restricted on their diet intake to the amount of food eaten by the group 2b rats.

Group 3b rats were given 400 mgm. of desiccated thyroid powder daily for 14 days. They were adrenalectomized on the 10th day and sacrificed for experiment on the 14th day after the start of thyroid administration. The group 3c rats were given 0.5 ml. of Wilson's adrenal cortical extract daily after they were adrenalectomized.

Succinic dehydrogenase and cytochrome oxidase were assayed in liver homogenates by methods developed by Schneider and Potter (2). The quantity of succinic dehydrogenase can be assayed by making it the limiting enzyme factor in the sequence: succinate—succinic dehydrogenase—cytochrome c—cytochrome oxidase—oxygen. Schneider and Potter (2) concluded from their data that cytochrome oxidase was in wide excess in most tissues, but under the experimental conditions used in this investigation we felt it necessary to assay the cytochrome oxidase activity at the same time as that of succinoxidase. If cytochrome c and cytochrome oxidase are in excess the succinic acid dehydrogenase is the limiting factor and the rate of oxygen consumption becomes a valid measure of dehydrogenase activity. Cytochrome c was added in excess. The high Q_{O_2} values for cytochrome oxidase indicate that the cytochrome oxidase is probably in excess in the experiments reported in this paper. This point is discussed in more detail later in the paper.

Cytochrome oxidase was measured by setting up conditions so that cytochrome c is reduced at a greater rate than it is reoxidized by cytochrome oxidase and oxygen. Ascorbic acid has proven superior to hydroquinone and other compounds as a reducing agent (2). The oxidase is made the limiting component in the system ascorbate-cytochrome c-cytochrome oxidase by adding an excess of cytochrome c. The autoxidation rate of ascorbic acid was obtained by using several concentrations of liver homogenate and extrapolating to zero tissue quantity. The rate of oxygen consumption was measured in Warburg manometers at 38°C. The cytochrome c was prepared by the method of Keilin and Hartree (5) but was left in aqueous solution (6).

The Q_{O_2} values are expressed as cubic millimeters oxygen per milligram dry weight per hour.

RESULTS. The liver tissue from the hyperthyroid rats showed a marked increase in the activities of the succinoxidase enzyme system and of the cytochrome oxidase as shown by the Q_{O_2} values of the 1a (control) rats and the 1b (thyroid-fed) rats in table 1. The succinic acid Q_{O_2} values rose 46 per cent while the oxidase values rose 46.5 per cent. The rats fed thyroid showed a significant decrease in weight over the 2-week period. As shown in figures 1 and 2 the Q_{O_2} rise appeared 4 to 6 days after the start of thyroid feeding and increased progressively for the duration of the test period. The 4th and 6th columns of table 1 show values of total liver Q_{O_2} obtained by multiplying the Q_{O_2} by the total dry weight of the liver computed on the basis of 100 grams of rat. These values are measures of the total dehydrogenase and oxidase activity in the whole

liver. The results given in table 1 show that the enzyme content of the whole liver also changes significantly in the hyperthyroid condition and after adrenalectomy. Changes in the total enzyme content give a more valid picture, perhaps, of the actual change in the whole liver of the treated animals than do the Q_{O_2} values alone.

Groups 2a, 2b and 2c in table 1 show the effects of adrenalectomy with and without treatment. Group 2a are controls on regular diet (Rockland rat pellets) restricted in quantity to the amount eaten by the group 2b rats. Group 2c

TABLE 1

*Succinoxidase Q_{O_2} and cytochrome oxidase Q_{O_2} of liver tissue of rats after thyroid feeding, after adrenalectomy, and after a combination of the two conditions**

GROUP NO.	NO. OF ANIMALS	SUCCINOXIDASE		CYTOCHROME OXIDASE	
		Q_{O_2}	$Q_{O_2} \times \text{Total dry wt.}$	Q_{O_2}	$Q_{O_2} \times \text{Total dry wt.}$
1a Control.....	18	97.5 \pm 3.50†	127,292 \pm 6353	367.1 \pm 17.8	477,590 \pm 8800
1b Thyroid.....	18	149.4 \pm 5.20	200,110 \pm 11,275	537.9 \pm 25.7	677,092 \pm 44,811
2a Control.....	12	112.4 \pm 6.50	141,918 \pm 8932	433.7 \pm 35.0	482,957 \pm 13,208
2b Adept.....	12	85.0 \pm 6.85	95,453 \pm 7906	303.0 \pm 35.5	343,993 \pm 51,676
2c Adept. treated.	5	105.0 \pm 7.50	102,500 \pm 8100	412.5 \pm 28.0	452,300 \pm 28,400
3a Control.....	11	99.0 \pm 2.70	132,012 \pm 5146	377.2 \pm 24.9	456,320 \pm 15,800
3b Adept.-thyr....	11	130.4 \pm 3.10	162,530 \pm 7604	468.4 \pm 14.0	553,400 \pm 13,400
3c Adept.-thyr. treated.....	6	151.0 \pm 8.70	172,400 \pm 5300	520.0 \pm 25.0	623,450 \pm 25,300

* Groups 1a, 2a, and 3a are control groups; group 1b rats fed 400 mgm. desiccated thyroid substance (Armour) daily for period of 14 days; group 2b rats adrenalectomized without treatment, sacrificed 7 days after operation; group 2c rats adrenalectomized, given 0.5 ml. Wilson's adrenal cortical extract daily, sacrificed 7 days after operation; group 3b rats fed 400 mgm. desiccated thyroid daily for 14 days, but adrenalectomized on 10th day; group 3c rats, same as 3b, but given 0.5 ml. of Wilson's adrenal cortical extract daily after operation, sacrificed 4-5 days after operation.

† Standard error of mean.

rats were given 0.5 ml. of Wilson's adrenal cortical extract per day until sacrificed for experiment on the 7th day after operation. As the table shows the average succinoxidase Q_{O_2} decreased 32 per cent after adrenalectomy, from 112.4 to 85; cytochrome oxidase also showed a significant decrease which confirms fairly exactly our earlier report using a somewhat different method of assay (1). The adrenal cortical extract prevented this decrease. Restricting the diet in normal rats appears to result in higher Q_{O_2} values than are found when the diet quantity is not restricted. The group 1a controls and the group 3a controls have lower Q_{O_2} values than do the 2a rats which were on restricted diet.

The differences between the mean Q_{O_2} of groups 1a and 1b, 2a and 2b, 3a and

3b and between groups 3b and 1b were tested for significance by Fisher's *t* test method. In all cases the probability was less than 0.01, showing that all of them are significant.

In order for this assay method to be a valid measure of succinic acid dehydrogenase there must be an excess of cytochrome oxidase present at all times. Otherwise the oxidase may be or become the limiting factor. As shown by the results in table 1, the ratio, cytochrome oxidase/succinoxidase, varies for the different groups from 3.31 to 4.02. Such a small variation in ratio raises some doubt about the independence of the succinoxidase Q_{O_2} values and the cytochrome oxidase values under our experimental conditions.

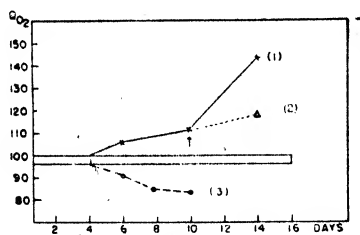


Fig. 1

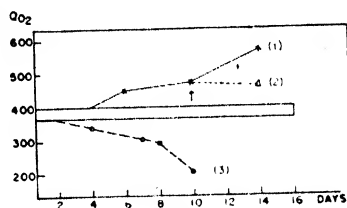


Fig. 2

Fig. 1. Changes in succinoxidase activity in liver tissue of rats receiving thyroid powder (1), of rats receiving thyroid powder and then adrenalectomy as indicated by arrow (2), and of rats after adrenalectomy (3). The horizontal block gives the range of variation of the control values.

Fig. 2. Changes in cytochrome oxidase activity in liver tissue of rats receiving thyroid powder (1), of rats receiving thyroid powder and then adrenalectomy as indicated by arrow (2), and of rats after adrenalectomy (3). The horizontal block gives the range of variation of the control values.

The coefficient of correlation, *r*, of cytochrome oxidase Q_{O_2} values to succinoxidase Q_{O_2} values in group 1a is 0.58; for 1b, 0.30; for 2a, 0.71; and for 2b, 0.94. Three of the four values are high enough to suggest some dependence between the two enzyme systems. The two enzyme systems vary independently of each other in group 1b. The high correlation in group 2b might be the result of some equal effect of a third common factor, the hormonal changes. Since the cytochrome oxidase Q_{O_2} values are high, above 300 in all group averages, there is probably an excess of cytochrome oxidase so the changes in succinoxidase measure real changes in the activity of the succinic acid dehydrogenase. By adding a standard oxidase to the sample of homogenate to assure a constant excess of cytochrome oxidase, the activity of succinic acid dehydrogenase could be assayed free from changes in cytochrome oxidase. Unfortunately we have not been able to do this yet.

The graphs in figures 1 and 2 indicate that the changes in Q_{O_2} after adrenalectomy do become significant on the 4th day to the 6th day after operation. Two rats were kept until the 10th day after adrenalectomy. They were obviously in much poorer condition but were not moribund. The succinoxidase Q_{O_2} ,

values were 51 and 53.4 while the cytochrome oxidase values had dropped to 115 and 53.4 respectively. As adrenal insufficiency became severe the cytochrome oxidase appeared to become the limiting factor in the succinoxidase reaction.

If rats which have been fed desiccated thyroid substance are adrenalectomized on the 10th day and maintained on salt solution as drinking water for 3 days, they almost invariably go into a crisis and are dead by the fifth day after operation whether thyroid substance is continued or not. The data on the group 3b rats as given in table 1 show that the Q_{O_2} values were higher than those of the controls on account of stimulation by thyroid hormone. However, the rise was significantly less than that shown by the liver tissue from group 1b rats. The deficiency in adrenal cortical hormones must contribute to the discrepancy since the values for the 3c rats which were kept on adrenal cortical extract after the operation showed values that do not differ significantly from Q_{O_2} values of normal rats fed desiccated thyroid. The inhibition of enzyme formation resulting from adrenal insufficiency is also shown graphically in the curves marked 3 in figures 1 and 2. Insufficiency of adrenal cortical hormones checked the increase in formation of respiratory enzymes resulting from thyroid feeding as shown in curves marked 2 in figures 1 and 2.

DISCUSSION. The results show that thyroid substance causes a marked increase in the succinoxidase enzyme system and cytochrome oxidase in liver cells. Since succinic acid dehydrogenase is probably a flavoprotein enzyme (7, 8) the action of the thyroid hormone may well be a general one, stimulating the manufacture of most or all of the oxidation enzymes. The increase in respiratory enzyme systems must be an important factor in the rise in oxygen consumption of the intact animal and of tissue slices following thyroid administration which has been observed by a number of investigators. The observation of a thyroid effect on d-amino acid oxidase, another flavoprotein enzyme, by Klein (9), and the indication by Gerard and Cohen (10) that thyroid substance increases oxidases and dehydrogenases in brain tissue of rats is in accord with the idea that the thyroid is a general respiratory enzyme stimulant. The hormone may stimulate the formation of enzyme protein by the liver as the observations of Klein (11) indicate, but it is highly probable that it affects the synthesis of prosthetic groups of some of the enzymes also.

The results presented in table 1 for the rats in groups 2 and 3 show that adrenalectomy results in a decrease in activity of the two enzyme systems. The cytochrome oxidase values confirm our previous report (1) and give it an additional significance since we are now using a more precise method of assay. Some substances from the adrenal cortex appear to regulate the activity of the succinic acid system and cytochrome oxidase. If the adrenalectomized rats are maintained on adrenal cortical extract the decrease in enzymatic activity does not occur. Since the deficiency in adrenal cortical hormones decreased the rise in enzymatic activity resulting from the thyroid stimulation as shown in the group 3 rats in table 1 and in curve 2 in figures 1 and 2, it appears that the adrenal cortex does have some part in the formation of oxidation enzymes in the liver cell. A longer test period after the adrenalectomy would probably have given more pro-

nounced results, but these animals do not survive more than 5 days after adrenalectomy. The strain of the severe hypoglycemia resulting from the combination of hyperthyroidism and adrenalectomy is probably a major factor in the short survival time.

The decrease in enzymatic activity after adrenalectomy hardly appears large enough to account for the general deterioration and death of the animal suffering from adrenal insufficiency. However, the changes that lead to death are gradual and over a period of days or weeks a small decrease in energy release in the cell may assume a magnitude all out of proportion to the changes observed in isolated enzyme systems.

SUMMARY

1. The activities of succinoxidase and cytochrome oxidase increased significantly in the liver tissue of rats fed desiccated thyroid substance. The enzyme changes could be demonstrated after the 4th day.

2. After adrenalectomy these enzyme systems decreased in activity.

3. The increase in activity of succinoxidase and cytochrome oxidase after thyroid feeding is not so great if the hyperthyroid rats are adrenalectomized during the period of thyroid feeding.

4. Five-tenths of a milliliter of Wilson's adrenal cortex extract per day appeared to prevent all the enzymatic changes produced by the adrenalectomy.

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RENAL BLOOD FLOW AND RENAL CLEARANCE DURING HEMORRHAGIC SHOCK¹

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Renal clearances have been studied by Corcoran, Taylor and Page (1, 2) during shock induced by hemorrhage and limb tourniquets in dogs, and by Lauson, Bradley and Cournand in traumatic and hemorrhagic shock in man (3). Phillips et al. (4) have investigated the adequacy of the renal clearance of p-aminohippuric acid (PAH) as a measure of effective renal plasma flow in dogs at normal blood pressure levels and during hypotension. All of these workers have qualified their interpretation of diodrast and PAH clearances as a measure of renal plasma flow during hypotension and shock with the premise that plasma extraction of these substances by the kidney must be almost complete to permit quantitative interpretation, viz., approximately 85 to 90 per cent (4, 5).

Efficient plasma extraction of PAH by the kidneys is maintained until the mean blood pressure following hemorrhage falls to about 60–80 mm. Hg in dogs (4). Diodrast extraction also apparently fails at blood pressure levels of about 60 mm. Hg (1), presumably because glomerular filtration pressure falls to 0, and urine formation ceases. Under these conditions, therefore, the plasma clearance of these substances will no longer accurately measure renal plasma flow. • Failure of diodrast extraction was also observed when blood was reinfused after hemorrhage and following limb tourniquet release (1, 2), probably the result of renal tubular impairment. Discrepancies between the clearance of creatinine and PAH and direct renal blood flow have been observed following complete ischemia of the kidney, accompanied by decrease in the renal extraction of PAH (6, 7).

In view of the probable alteration of the mechanism of renal clearance in shock resulting from hypotension and anoxia, it appeared important to investigate renal blood flow by a direct method under these conditions. Simultaneous renal clearance of creatinine and PAH made it possible to evaluate the adequacy of these measurements in hemorrhagic shock by comparison with direct renal blood flow measurements.

METHODS. The dogs used in these experiments were anesthetized with 30–35 mgm. per Kg. of pentobarbital sodium given intravenously. The left kidney was approached usually by a ventral, occasionally by a flank incision, surgical bleeding being carefully controlled by ligation and electro-cautery. The left ureter was cannulated close to the pelvis of the kidney to minimize dead space in the collection system, and a loose ligature was placed around the renal vein, preparatory to cannulation. Femoral arteries were cannulated for bleeding and

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registration of mean blood pressure. A femoral vein was prepared for infusion of saline containing creatinine and PAH. Both external jugular veins were exposed for cannulation.

The method for measuring whole renal blood flow directly has been described in detail in a previous report (7). In brief, a special thin-walled cannula, 46.5 cm. long, was passed down the right external jugular, vena cava, and into the renal vein. Continuous saline infusion through the cannula kept it free of blood until the dog was heparinized with 40 mgm. per kgm. of body weight (additional heparin was given at the rate of 50 mgm. total dose every half-hour). The renal cannula was then securely tied, and renal vein blood was allowed to flow immediately through the cannula to the exterior, then reinfused directly into the opposite external jugular vein by a short connecting tube. A T-cannula in the latter allowed renal vein blood to be shunted periodically into a 10 cc. graduated cylinders, the outflow being accurately timed with an electric chronometer. From this, whole renal blood flow (RBF) in cubic centimeters per minute was directly calculated. The renal vein blood aliquots were then immediately reinfused.

Continuous infusion of creatinine and PAH was used to maintain approximately constant plasma levels, that of PAH being kept usually below 2.0 mgm. per cent. Infusion was discontinued during the 60 and 40 mm. Hg periods of hypotension, because of anuria and cessation of renal excretion. It was begun again with reinfusion of blood following the 40 mm. period. To determine clearances, arterial plasma samples were taken during each urine collection period, and were analyzed for creatinine by the alkaline-picric acid method (8), and for PAH by a modification of the method of Bratton and Marshall (9). Plasma proteins were precipitated by the CdSO_4 method (10). Hematocrit determinations were made by spinning each arterial blood sample at 2,000 rpm in Wintrobe hematocrit tubes. A correction factor of 0.96 was applied for the plasma trapped between the cells (11). Between the time of renal vein cannulation and beginning of the control urine periods, a moderate amount of saline was infused (16 cc. per kgm. of body weight) to aid in sustaining the urine flow.

Hemorrhagic shock was produced by the standardized technique developed in this laboratory (12). This consists of bleeding rapidly to a mean blood pressure of 50 mm., maintained for 90 minutes, then bleeding to 30 mm., and sustaining pressure at this level for 45 minutes. Then the warmed, filtered blood is rapidly reinfused; if irreversible shock has been established, as happens in most dogs under these conditions, the animal dies after a variable period of time from circulatory failure.

Because of the added insult of the surgical procedures, the above procedure has been slightly modified, in that the dogs were bled to a mean blood pressure of 60 mm., and then to 40 mm., instead of the customary 50 and 30 mm. periods. That this treatment has been adequate to produce the desired irreversibility is evidenced by the fact that 5 of the dogs observed died precipitantly. The remaining eight animals observed survived for longer periods following the rein-

fusion; two died during the third hour of the post-infusion period, and the remainder were sacrificed when the blood pressure had again fallen to 50–60 mm. This was done to minimize the effect of additional anoxia on the kidneys, since it was desired to examine them histologically for the effects of the initial periods of hypotension. Typical hemorrhagic intestinal changes were noted in all dogs, but were variable in degree.

RESULTS. A. *Changes in direct renal blood flow.* In analyzing the changes in RBF, it is desirable to classify the dogs on the basis of their survival. Accordingly, in table 1 appear 8 experiments in which the animals survived more than two hours after the reinfusion of blood, with adequate return of mean blood pressure and RBF soon after reinfusion. The results of these eight experiments are graphically summarized in figure 1. Of the remaining five animals, two died before the conclusion of the 40 mm. period, two died within 35 minutes after the reinfusion of blood, without adequate return of mean blood pressure and RBF, and the fifth survived for several hours after reinfusion, but was atypical in that RBF remained continuously reduced. This latter group will be referred to as the group of "limited survival".

Reference to table 1 shows that RBF determinations made every minute or two during the control period averaged 160 cc. per minute per kidney (range, 117 to 211); the control period averaged 45 minutes in length. Another series of RBF observations were made early in the 60 mm. period, and these averaged 66.5 cc. per minute (range, 26 to 89). Late in the 60 mm. period RBF averaged 32.4 cc. per minute (range, 9 to 66.5). During the 40 mm. period, RBF averaged even less, 18 cc. per minute per kidney (range, 9 to 35.5).

During the first hour after reinfusion of blood, blood pressure recovered to a mean value of 101 mm. Hg, compared to the control value of 132 mm. The RBF in the experiments of table 1 showed a corresponding recovery, averaging 107 cc. per minute per kidney (range, 66 to 145). Then, as the mean blood pressure declined to an average of 80 mm. during the second hour after reinfusion, RBF averaged 62 cc. per minute (range, 17 to 93). During the third post-infusion hour, mean blood pressure averaged 56.5 mm., and RBF was only 25 cc. per minute (range, 15–31).

In order to permit grouping the data with greater uniformity, RBF and clearances are presented in figures 1 and 2 in terms of cubic centimeters per minute per gram of kidney weight. In figure 1, the RBF is plotted in a curve which represents the arithmetic mean of the observations made in the eight experiments of table 1.

Reduction in circulating blood volume following the initial bleeding was the cause of the immediate decrease in RBF. However, compensatory mechanisms come into play, and additional small quantities of blood must be withdrawn to maintain the animals at the 60 mm. level (see curve showing per cent of hemorrhage, fig. 1). A factor contributing to this compensatory rise in blood pressure may be increased vascular resistance in various vascular beds. The kidney apparently contributes to this increased peripheral resistance as evidenced by the

TABLE 1

The changes in renal blood flow, renal vascular resistance, and mean blood pressure during hemorrhagic shock in dogs which survived for several hours after reinfusion of blood

EXPT. NO.	CONTROL	60 MM. PERIOD		40 MM. PERIOD	HOURS AFTER REINFUSION		
		Early	Late		1	2	3
1. MBP*.....	134	51.5	50.5	28.5	98	63	
RBF†.....	170	61	21	15	93	46	
PRU‡.....	0.79	0.845	2.45	1.90	1.05	1.37	
2. MBP.....	137	59	57	30	100	88	54
RBF.....	125	26	9	15	93	85	31
PRU.....	1.10	2.27	6.30	2.00	1.08	1.03	1.74
3. MBP.....	135	51.5	61.5	42	130	127	62.5
R.B.F.....	165	87	31	9	123	82	19.5
PRU.....	0.82	0.60	2.00	4.65	1.06	1.55	3.20
4. MBP.....	133	56	53	35	92	54	
RBF.....	121	40	27	16	90	25.5	
PRU.....	1.10	1.40	2.00	2.20	1.02	2.12	
5. MBP.....	137	58	59	36	107	90	55
RBF.....	177	82	40	22	137	(no observations)	15
PRU.....	0.78	0.71	1.50	1.64	0.78		3.66
6. MBP.....	95	58.5	60	40	91	64	
RBF.....	117	89	45	21.5	66	17	
PRU.....	0.815	0.66	1.34	1.85	1.38	3.80	
7. MBP.....	139	58	49	34.5	79	74	52
RBF.....	209	75	66.5	35.5	112.5	85	29
PRU.....	0.67	0.78	0.735	1.03	0.694	0.88	1.77
8. MBP.....	148	59	60	40.5	109	93	59
RBF.....	211	72	20	9.4	145	93	31
PRU.....	0.70	0.82	3.00	4.30	0.75	1.00	1.91

* MBP: Mean arterial blood pressure in mm. Hg.

† RBF: Whole renal blood flow in cc./per min./per kidney, measured directly. Average kidney wt. in this series was 38 gms.

‡ PRU: Peripheral resistance units, obtained from the ratio $\frac{\text{MBP}}{\text{RBF, cc./min.}}$.

increased PRU.² From a control value of 0.843 PRU, the renal resistance increases to 1.12 PRU early in the 60 mm. period to 2.3 PRU late in that period.

² We employ the designation of Green (13) for peripheral resistance, where a "peripheral resistance unit" (PRU) is defined as being equal to $\frac{1 \text{ mm. Hg.}}{1 \text{ ml./min.}}$. Total renal resistance is determined by taking the mean arterial blood pressure over RBF: $\frac{\text{MBP, mm. Hg.}}{\text{RBF, cc./min.}}$. Renal venous pressure is taken as zero.

This trend continued during the 40 mm. period to reach an average value of 2.7 PRU. Upon reinfusion of blood, the renal resistance returns approximately to normal, 0.95 PRU, gradually increasing again terminally to 1.16 PRU and 2.47 PRU during the second and third hours after reinfusion. (The average PRU values are plotted in fig. 1, where they may be related to changes in RBF and mean blood pressure).

Although data are limited, it is of interest to compare with the above findings the changes in the group of animals of limited survival. Control RBF averaged 190 cc. per minute per kidney (range, 139 to 319) at a mean blood pressure of 128 mm. Hg. During the 60 mm. period, RBF was much less than in the above group, averaging 39 cc. per minute (9.5 to 73) early in the period, and only 17 cc. per minute (7 to 27) late in the period. This trend was further accentuated

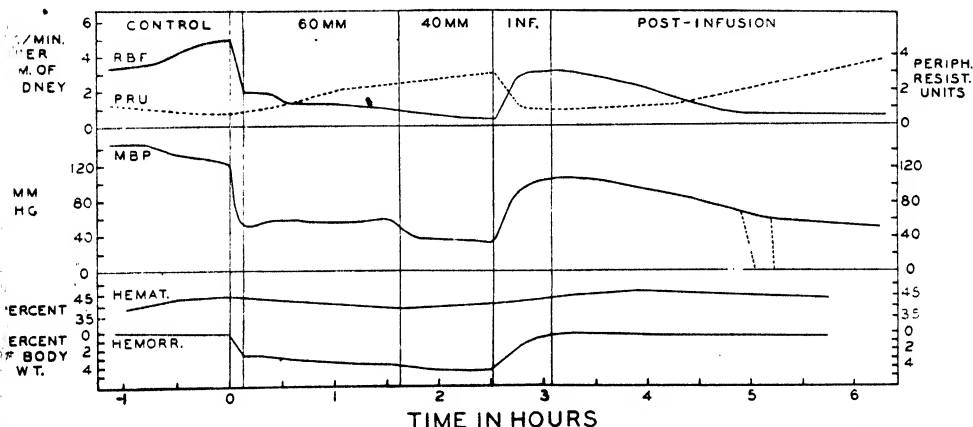


Fig. 1. The changes in direct renal blood flow (RBF), renal vascular resistance (PRU), and mean blood pressure (MBP) in the course of hemorrhagic shock. Each line represents the arithmetic mean of the trend of a total of eight experiments in which survival extended into the post-infusion period.

in the 40 mm. period, where RBF averaged only 6.5 cc. per minute. In three of these experiments, RBF ceased entirely during the 40 mm. period.

As might be expected, the renal resistance during hypotension was greater in the group of limited survival than in the first group of animals. While the control value was 0.724 PRU, early in the 60 mm. period the renal resistance was 2.62 PRU, increasing to 4.62 PRU late in this period. Since renal blood flow stopped entirely during the 40 mm. period in three of the animals, renal vascular resistance accordingly by definition increased to infinity. In two animals in which observations were made in the post-infusion period, RBF was only 20 and 47 cc. per minute respectively, and the renal resistance averaged 2.54 PRU.

It is apparent, therefore, that in animals of limited survival, RBF is considerably lower and renal vascular resistance is correspondingly higher than in animals of more prolonged survival. How this may be related to the severity of shock cannot be definitely stated. It is possible that increased vascular resistance in the kidney may increase the severity of anoxia in that organ.

B. Changes in the renal clearances. Complete renal clearance changes could of necessity be followed only in animals which survived beyond the time of reinfusion. Therefore, experiments exemplifying the changes in clearance during hemorrhagic shock have been taken from the animals in table 1. Of these, five experiments have been selected to show the representative trend of events, and are presented individually in figure 2. Of the remaining three experiments, one (expt. 6) was not satisfactory because the plasma concentration of PAH was inadvertently elevated during the control clearance, causing self-depression of the clearance. In two others, experiments 7 and 8, there was an oliguria of less than 0.1 cc. per minute following the reinfusion of blood, making it impossible to compare clearances with RBF with any degree of accuracy, since under these circumstances a considerable time lag is involved between the formation of urine at the glomeruli and the time it is collected.³ In the experiments of figure 2, urine flow has been adequate to allow comparison of clearances and simultaneous RBF. The time of the clearance periods has been corrected for emptying time, approximated from the rate of urine flow and the estimated dead space of the collection system.

1. *Control RBF and clearance.* When the clearance of PAH is referred to in this report, unless otherwise specified, it will imply the total volume of the plasma clearance plus the hematocrit volume, or in brief, renal blood flow derived from the PAH clearance. This is designated as BF_{pah} , to distinguish from the direct renal blood flow measurements, RBF. (In fig. 2, BF_{pah} has been simply designated as "PAH").

During the control periods of figure 2, BF_{pah} averages 140.5 cc. per minute, while RBF averages 148 cc. per minute. The ratio of BF_{pah}/RBF is thus 0.95 (range, 0.88 to 1.11). In a larger number of experiments reported elsewhere (7), the ratio BF_{pah}/RBF averaged 0.91. The clearance of creatinine averaged 23.0 cc. per minute per kidney (range, 16.5 to 30) in the present control periods.

2. *Sixty and 40 mm. periods.* When the blood pressure was reduced by hemorrhage to approximately 60 mm., urine formation was found to cease in these experiments. Sometimes traces of urine were recovered, but analysis was unreliable, for in all probability such samples were contaminated with urine formed during the control periods, remaining stagnant in the tubules and collection system during the period of hypotension. However, in most instances, there was complete anuria, and hence the clearances were taken to be "0". Thus, while the apparent renal blood flow based on the clearance of PAH would suggest complete renal anemia, actually 66.5 cc. of blood per minute would be passing

³ During a 90 minute period after reinfusion of blood, RBF in experiment 7 averaged 99 cc. per minute, and urine collected during this time gave a value for BF_{pah} of only 10.5 cc. per minute, while creatinine clearance was 0.6 cc. per minute. In experiment 8, RBF averaged 124 cc. per minute during a 105 minute period of observation, and during this time BF_{pah} averaged 8.0 cc. per minute, and the creatinine clearance was 2.0 cc. per minute.

It must be stated again that these values of RBF and clearances cannot be directly compared because of the large lag between the time RBF is measured and the time the urine is collected. These experiments nevertheless emphasize the large disparities which may result when the kidney is subjected to prolonged hypotension and anoxia.

through the kidney early in the 60 mm. period. This error is reduced as RBF decreases during this period as the result of increased renal resistance, and is less during the 40 mm. period, when the measured RBF is only 11 per cent of the control flow.

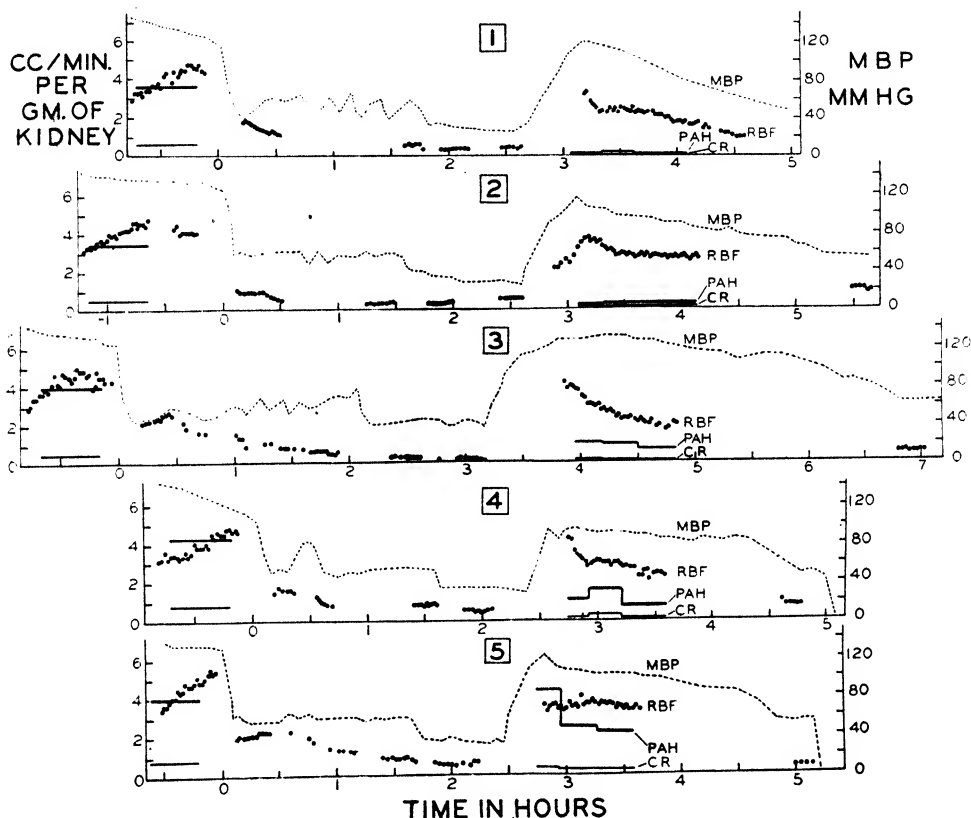


Fig. 2. Comparison of RBF and renal clearances in five representative experiments. Broken line (MBP): mean blood pressure; solid circles (RBF): direct whole renal blood flow. Upper solid line (PAH): the renal blood flow calculated from the plasma clearance of PAH, plus the hematocrit volume. Lower solid line (CR): clearance of creatinine. Kidney weights in the above experiments were as follows: no. 1, 42.5 grams; no. 2, 31.5 grams; no. 3, 39 grams; no. 4, 31 grams; no. 5, 38.5 grams.

3. *Post-infusion period.* Following an average interval of 28 minutes from the beginning of reinfusion of the blood, during which time urine was discarded, clearance observations were resumed and continued for three periods. In figure 2 these experiments have been arranged in order of the severity of the effects on the renal clearances. It may be seen at a glance in figure 2 that BF_{PAH} is typically reduced below RBF, varying from 9 to 85 per cent of simultaneous

RBF, and averaging 39 per cent. This is a 61 per cent error in the calculation of renal blood flow when based on PAH clearance. The creatinine clearance decreases from 23.0 cc. per minute to 5.4 cc. per minute, a decrease of 77 per cent. Since RBF decreases only 29 per cent and mean blood pressure averages 107 mm. in this group, it seems plausible that the clearance of creatinine no longer measures glomerular filtration rate, for in the face of adequate renal blood flow and blood pressure one would not expect the actual filtration rate to be depressed as much as indicated.

Considering in addition the results of experiments 7 and 8, in which there was extreme oliguria in the post-infusion period (see footnote 3), even while mean blood pressure and RBF remained fairly adequate, it must be emphasized that the net effect of hemorrhagic hypotension on the clearances is even greater than the above figures would indicate.

4. *Effect on the extraction ratio of PAH.* The average of 17 control observations in different experiments was 0.73 (0.635 to 0.935).⁴ A group of 8 observations made late in the 60 mm. period were extremely variable, ranging from -0.650 to 0.543; of these eight, 5 gave "negative" extraction ratios (i.e., the concentration of PAH was higher in the renal vein blood than in the systemic arterial blood), and the average of all eight was -0.0234. Four out of 5 observations made during the 40 mm. period gave negative extraction ratios; the average of all five was -0.72 (-1.50 to 0.285). Thus it is seen that extraction ratios are typically negative late in the 60 mm. and during the 40 mm. period, 9 of the 13 observations being negative, and all averaging -0.29. Plasma concentrations of PAH for all these observations ranged between 0.21 and 1.66 mgm. per cent.

When the blood was reinfused there was a return of the extraction ratio toward normal, 9 observations during the earlier clearance periods after reinfusion averaging 0.406 (0.03 to 0.69). During the later clearance periods after reinfusion the ratio began to fall off again, averaging 0.213 for 10 observations (-0.056 to 0.67). The plasma concentrations ranged from 1.0 to 3.2 mgm. per cent during these observations.

5. *Effect on U/P ratio of creatinine.* The control U/P ratio in the five experiments of figure 2 averaged 76.5 (129 to 26) at an average urine flow of 0.4 cc. per minute per kidney (range, 0.2 to 0.645). Observations made during the post-infusion period show a decrease of U/P ratio to 15.5 (33 to 4.1), while the urine flow, though variable, averaged the same, 0.4 cc. per minute (range, 0.126 to 0.8).

6. *Histological changes.* Histological changes were concerned with the renal

⁴ Extraction ratio is defined as $\frac{A-V}{A}$, where A and V are the arterial and renal vein plasma concentrations respectively in milligrams per cent. The data on extraction ratio in this report must be qualified by the fact that due to technical difficulties, renal vein blood samples could not be immediately centrifuged. This allows some of the PAH which has penetrated the RBCs to diffuse into the plasma (4, 14), giving extraction figures somewhat lower than data published by Phillips et al. (4). However, experimental changes in extraction ratio have been so extreme that greater quantitative accuracy should not invalidate the conclusions drawn.

tubules, which exhibited a mild, diffuse nephrosis with cloudy swelling and hydropic degeneration of the tubular cells. Casts appeared in some of the tubular lumens. Although the morphological changes were minimal, the proximal convoluted tubules seemed to show the more marked effects. The damage in the kidneys examined was variable, and could not be directly related to the degree of functional change.⁵

DISCUSSION. Data on cardiac output in dogs obtained by H. C. Wiggers (15) indicate that the expected cardiac output for the average size of dogs used in these experiments would be 1710 cc. per minute. Control RBF in all 13 experiments averaged 172 cc. per minute per kidney (or 344 cc. per minute for both kidneys), a value of 20 per cent of the expected cardiac output. During the hypotensive periods of standardized hemorrhage, cardiac output in dogs falls to 29 to 45 per cent of the control, hence to an expected cardiac output of 630 cc. per minute. The total RBF for both kidneys would be 28 cc. per minute during the 40 mm. period, or now only 4.5 per cent of the cardiac output. This supports the conclusion of Lauson, Bradley, and Cournand (3) that in hemorrhagic hypotension, blood is shunted away from the kidney presumably to other parts of the body, and that this effect results from increased renal vascular resistance. Although the hematocrit falls during hypotension in these dogs (see fig. 1), probably indicating an actual decrease in blood viscosity, the PRU value nevertheless rises. This increase begins during the 60 mm. period, permitting the tentative conclusion, based in part on other evidence, that the increase in renal vascular resistance results in part from vasoconstriction.

We are unprepared to state whether the apparent renal vasoconstriction during the hypotensive periods is due to nervous or humoral mechanisms. Clamping of the renal artery results in maintained renal vasoconstriction which seems best explained by a humoral mechanism (7). One might conjecture that the reduced RBF during hypotension would create a situation analogous to renal ischemia. In fact, the gradual onset of increased renal vascular resistance during the 60 mm. period suggests a humoral mechanism. Contrary to this interpretation, however, is the behavior of RBF immediately after reinfusion of blood. Combined with adequate restoration of blood pressure and RBF, there is a reduction of renal vascular resistance to approximately normal. If a humoral mechanism were operative, continually reduced renal blood flow might be expected after reinfusion. Indeed, such an erroneous conclusion could result if one were to base his deduction on the BF_{pah} data presented in figure 2, which shows the renal blood flow much lower than the actual renal blood flow as measured by the direct method.

The disparity between renal clearances and RBF during the post-infusion period is believed to be the result of renal tubular impairment created by anoxia during the period of hypotension. Tubular excretory impairment is indicated by the reduced extraction ratio of PAH, and this impairment is roughly correlated with the degree of disparity between BF_{pah} and RBF early in the post-infusion period (see fig. 2). In experiment 1, the extraction ratio is 0.250; in experiment

⁵ We are indebted to Dr. Harry Goldblatt for examination of the histological specimens.

2, 0.165; in experiment 3, 0.400; in experiment 4, 0.635; and in experiment 5, 0.683.

Comment should be made on the "negative" extraction ratios observed during the late 60 mm. and during the 40 mm. periods. Corcoran and Page observed low and even negative extraction ratios for diodrast and inulin in some of their experiments during hypotension following hemorrhage (1). These negative values they attribute to concentration of these substances in the renal interstitial fluid, and a return from here to the renal vein blood when the urine flow and tubular excretion have virtually ceased during hypotension. This explanation is reasonable when it is kept in mind that the return of even relatively small quantities of PAH from the interstitial fluid to the renal vein blood might be expected noticeably to elevate the PAH concentration in the small volume of blood moving through the kidney at this time.

The change in the U/P ratio of creatinine is attributed to tubular reabsorption of this substance. This seems particularly likely when it is noted that marked decreases in U/P ratio occur in the post-infusion period even while the urine volume is reduced below the control value. Shannon and Winton (16) have found that the clearance of creatinine is significantly lower than that of inulin in perfused dog kidneys which have been subjected to variable degrees of anoxia during transfer from the animal to the perfusion apparatus. This reduced clearance of creatinine apparently resulted from tubular reabsorption of filtered creatinine. The more efficient clearance of inulin under these circumstances suggests that it may serve as a more reliable index of glomerular filtration during conditions encountered in shock.

The changes in urine flow following the reinfusion of blood have been extremely variable, being either greater or less than the control flow. This is contrary to the effects of complete renal ischemia, when the urine flow is invariably reduced following the release of the arterial clamp (7). Although hyposthenuria results in the present series, as indicated by smaller concentrations of PAH and creatinine relative to urine volume, nevertheless the effects of hemorrhagic anoxia do not appear to be as severe as the effects of complete renal ischemia. Following the latter, there is hyposthenuria combined with oliguria. A parallel sequence of events is seen following uranium poisoning in the dog (17). Here milder degrees of poisoning produce hyposthenuria without oliguria; more severe poisoning produces both hyposthenuria and oliguria, the glomerular filtrate being completely reabsorbed by the tubular cells. The tubular cells now act like a "dead" membrane, and the absorbing force is the osmotic attraction of the peritubular blood colloids.

SUMMARY AND CONCLUSIONS

When mean blood pressure in dogs is reduced to 60 mm. Hg by bleeding in the course of a standardized procedure used in producing hemorrhagic shock, direct renal blood flow decreases immediately to 41.5 per cent of the control value in typical experiments. As this level of hypotension is maintained, there is a gradual increase in renal vascular resistance, resulting in further decrease of renal blood flow. Minimal renal blood flow occurs during the 40 mm. period,

where it is only about 11 per cent of the control value. During this period, renal vascular resistance is greatest.

Upon reinfusion of blood there is an immediate, though not complete, restoration of renal blood flow and mean blood pressure to approximately 70-80 per cent of the control figures. Renal vascular resistance decreases simultaneously to approximately the control value. Terminally, blood pressure and renal blood flow decrease again, but renal blood flow falls more rapidly than the simultaneous mean blood pressure, due to increased renal vascular resistance.

In a group of dogs which died precipitantly, total renal blood flow on the whole is less during hypotension and a limited post-infusion survival period, and renal vascular resistance is correspondingly greater.

During the periods of hypotension there is anuria, and the clearances of p-aminohippuric acid (PAH) and creatinine are therefore zero, even while appreciable amounts of blood are flowing through the kidney.

Upon reinfusion of blood, the clearance of PAH and creatinine fail to be restored, resulting in large disparities between blood flow based on the PAH clearance, and the direct renal blood flow measurements. The creatinine clearance also loses its value as a measure of glomerular filtration, for it remains reduced to a far greater extent than could be expected with the existing conditions of blood pressure and renal blood flow.

These discrepancies are thought to be due to renal tubular impairment, created by anoxia during the hemorrhagic hypotension. This is evidenced by the reduction in the extraction ratio of PAH, signifying loss of tubular excretory ability, and by the marked decrease in the U/P ratio of creatinine, even with decrease in the urine volume, suggesting tubular reabsorption of the filtered creatinine. A mild, diffuse nephrosis characterized by cloudy swelling and hydropic degeneration of the tubular cells accompanies the functional alteration.

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THE INITIATION OF SWEATING IN RESPONSE TO HEAT¹

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Sweating occurs over most or all of the body surface of a man who enters a hot atmosphere. Upon the promptness with which sweating begins might conceivably depend his ability to tolerate heat. Insofar as sweating is aroused by reflexes from the body surface, it might start within a few seconds of exposure to heat. But if the whole body is required to build up an excess of heat within it before sweating commences, a considerable delay may be encountered.

Measurements were therefore undertaken to find how long a time is required for sweating to commence and for the sweating to accelerate to its maximal rate. In the past, rates of evaporation of sweat over local areas of body surface have been ascertained; they indicate that 5 to 20 minutes may be consumed in initiation (Kuno, 1934; Pinson, 1942). In the present study, rates of evaporation from the whole body were investigated.

METHOD. A Sauter balance was set up in the hot room. This balance is sensitive to 0.1 gram when holding an inanimate load. When holding a man, such sensitivity does not prevail in ordinary weighing, for the man is losing weight and his minimal movements introduce disturbances. But if the balance is kept swinging, and all the successive swings are read on its pointer, a sensitivity of about 0.2 gram is attained with a slowly changing (<1 gram per min.) load (fig. 1). The sensitivity is reduced to 0.6 gram with a load that changes by 5 grams per minute. The method consists therefore in reading the extremes of all swings, whose times of occurrence are noted to the second, and finding the mid points between simultaneous virtual extremes. The rate of evaporative loss is a difference between two mid points; determinations from a single swing may err by 0.4 gram to 1.2 grams, but successive determinations may be averaged to yield more reliable weight decrements.

When loaded, the balance had a swing period of 30 to 34 seconds. Mid points were therefore computed at those intervals of time. From them slopes were drawn to represent the succession of body weights. Slopes were then read off at intervals of approximately one minute, and when plotted they indicate the rates of loss at diverse times of exposure.

A subject, clad only in jockey shorts, entered the hot room at 0800 o'clock and sat in a light, wide-meshed, wicker chair suspended on the Sauter balance. Two to five minutes were required to counterbalance the man and obtain the first readings of pointer position. As the man lost weight the swings were pre-

¹ The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Rochester. The Sauter balance was generously loaned by the Department of Pediatrics of the University of Rochester.

dominantly to one side, whereupon a weight of 10 or 20 grams was added to the man. The pointer was thereby displaced and in a new series more mid positions were ascertained. At the same time the calibrating deflection of the mid position of the pointer showed an equivalence to the added weight. In 49 such calibrations it was found that 1 gram equals 1.08 scale divisions traversed by the pointer, with a standard error of ± 0.012 division. They did not differ for the two subjects tested nor with the amount of deflection of the pointer that was produced.

The chief condition for validity of the measurement was that all sweat should evaporate as soon as it was secreted by the sweat glands. This prompt evapora-

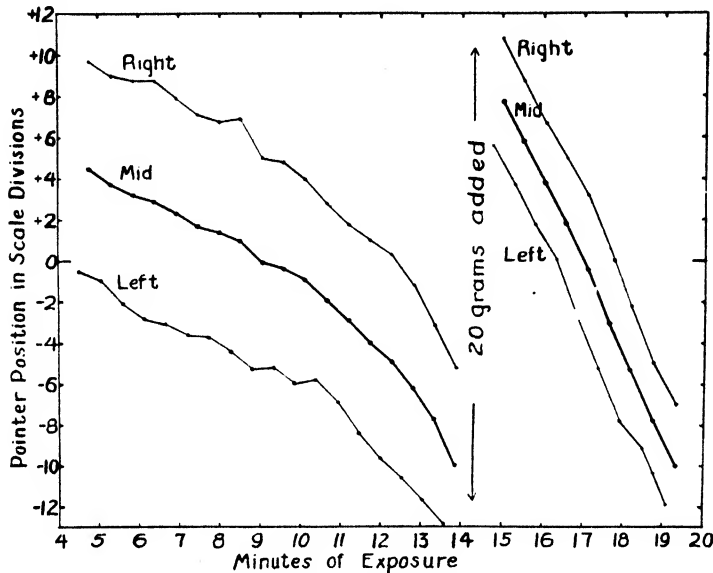


Fig. 1. Time course of balance swings during measurement of evaporative loss of body weight in subject D, 29 Jan.

tion was secured by having the atmosphere hot and dry (122°F ., 10 per cent relative humidity). The room was not equipped to exclude radiation or gradients of air temperature.

In this atmosphere, minute droplets of sweat can be seen to exude suddenly at one pore after another of the skin, disappearing during 5 to 20 seconds following. This succession of events is an ocular demonstration of the well-known intermittency of sweat outpouring by any one gland (Kuno, 1934, p. 216).

The results were obtained by the careful co-operation of E. J. Towbin, R. W. Stanley, F. W. Hastings and R. S. Dick.

RESULTS. Eight tests in a temperate atmosphere (76°F ., 30 per cent R.H.) demonstrated that evaporative losses were appreciably constant during periods of 5 to 14 minutes. The mean rates of loss were 0.42 gram per minute in subject

D and 0.64 in subject H, a significant difference. No attempt was made to standardize the subjects further. It is well known that of this minimal loss, up to one-tenth may represent loss of carbon (CO_2 minus O_2), and that the remainder is loss of water by evaporation.

The initial rates of loss observed, after the nude subjects left the control room (83°F ., 24 per cent R.H.) and entered the hot room, were greater than those in the temperate atmosphere by $2\frac{1}{2}$ fold (1.0 gram per minute in D and 1.8 in H). This result probably indicates that a first acceleration of sweating over that in the pre-exposure atmosphere has occurred within the first five minutes of exposure. Yet in most tests a new uniform rate of loss prevailed before the rapid second acceleration now to be described.

At the end of 12 minutes in subject D and of 7 minutes in subject H, rates of evaporative loss increased rather sharply (fig. 2). From only 1 to 2 grams per minute they increased to 4 to 6 grams per minute. The times at which appreciable second acceleration has occurred were read from time graphs; the indi-

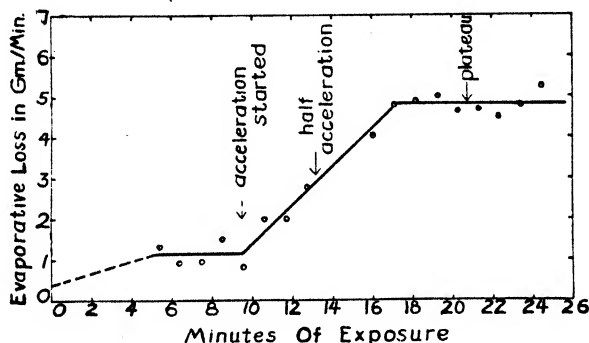


Fig. 2. Time course of rate of evaporative loss in subject D, 29 Jan.

vidual tests are listed in table 1. The difference in time of onset of faster rates was consistent between the two subjects.

Thereafter the rates increased rapidly up to a plateau (fig. 2). These rates were reached in about 20 minutes; in this respect the subjects did not differ appreciably. Often the plateau rates were not reached sharply but rather gradually.

Perhaps the most convenient measurement of time required for onset of sweating was the attainment of half acceleration, that is, of half the plateau rate minus the initial rate. This point of time clearly differed in the two subjects, being 15 minutes in D and 9 minutes in H.

Hence sweating was initiated much sooner in H than in D. Since no differences in their control states are known other than that in their minimal rate of evaporative loss, the diversity can be described as a contrast in their responses to heat, in their reflexes to the sweat glands. H regularly got his sweat glands into rapid action in half the time that D did.

It might be supposed that H was thereby more tolerant to heat. Actually the reverse was the case; H endured the hot atmosphere for only $6\frac{1}{2}$ hours in each of his first three exposures, while D and six other subjects were able to remain exposed during the 8 hours as planned. Promptness of sweating was therefore not a sign of tolerance to heat.

During the successive exposures that induced acclimatization to heat, no change in the time of onset of sweating was evident in either of the two subjects.

The rate of sweating that prevailed after 20 minutes of exposure (plateau rate) was 4 grams per minute in D and $5\frac{1}{2}$ grams per minute in H. The difference is surprisingly large for two men of equal size. These rates were somewhat less than the average rates for either subject during the remainder of the day's

TABLE 1

Rates of evaporative loss and times after exposure to heat at which they prevailed

DATE	MINIMAL RATE	PLATEAU RATE	ACCELERATION FROM MINIMAL RATE	AT HALF OF PLATEAU RATE	ATTAINED PLATEAU RATE
Subject D, age 28, 66 kgm., 1.79 m. ²					
	gm./min.	gm./min.	min.	min.	min.
11 Jan.	1.2	4.2	12	14.5	26
15	1.1	3.3	16	18.5	25
18	0.9	4.5	12	14.5	20
22	1.0	4.2	13	19.9	23
25	0.9	3.3	11	12.5	18
29	1.1	5.2	10	13.5	17
Mean of 6.....	1.0	4.1	12.5	15.4	21.5
Subject H, age 26, 63 kgm., 1.82 m. ²					
	gm./min.	gm./min.	min.	min.	min.
12 Jan.	1.9	5.2	5	12	20
16	—	6.6	8	8	20
19	1.7	5.8	7	8.5	21
23	2.2	4.9	8	9	14
Mean of 4.....	1.9	5.6	7	9	19

exposure (6.3 and 7.4 gram/min. respectively). But during the day various postures were required and diverse routine tests were performed, with the result that physiological states were not constant. Subject H in fact did short bouts of exercise every hour. It is probable that the plateau rate would have prevailed throughout eight hours of exposure if the subject had remained on the Sauter balance.

Observations were made on each subject to ascertain whether the rate of sweating fluctuated, once maximal rate had been obtained. In one series of 8 periods, rates seemed to vary between 5.5 and 9.2 grams per minute. The fluctuation was real, but what it represents is debatable, for the following reasons.

a. The subject had finished a bout of intensive exercise only 20 minutes before

the observations began. *b.* Some visible sweat remained on the skin, and the conditions for its evaporation may have varied. *c.* The routine of observations in all cases involved changes of posture and of exposure as other tests were performed.

If the question of fluctuations were based entirely upon the aftermath of first exposure, as exemplified in the last portion of figure 2, fluctuations of probable significance were also observed. It seems safer to conclude, however, that it is not yet known whether the rate of sweating for the whole body varies from minute to minute during steady sweating.

COMMENT. The initiation of full sweating is delayed by 5 to 15 minutes after hot air impinges upon the skin. This delay is too long to represent reaction time; hence it is likely that the actual stimulus is coming to be effective during the period. The skin starts to warm at once, but that stimulus alone speeds up the rate of evaporative loss by $2\frac{1}{2}$ fold at most. The greater delay suggests that the body as a whole accumulates a certain amount of heat before sweating is profuse.

It is well known that local heating produces local sweating. The above data indicate that the response to local heating is restricted; sweating becomes profuse only when the body is sufficiently warm that no more heat can be utilized in warming distant regions of the man. The first stimulus, yielding $2\frac{1}{2}$ fold sweating, may be the warming of the skin; the second stimulus, yielding 10 fold sweating, may correspond to the total excess of heat in the whole body. Possibly an increase of deep temperature is concerned in the second stimulus; we estimate, by interpolation of many measurements of rectal temperature, that an increase of less than 0.2 Centigrade degree has occurred at the time of second acceleration of sweating. This agrees with approximate data of Dantas (1939) who placed men under rather different atmospheric conditions.

The delay in acceleration of sweating certainly varies with the atmospheric temperature to which men are exposed. In a dry-bulb temperature at 95°F. the onset requires much longer than in 122°F. In comparing atmospheres it seems likely that both the first stimulus and the second stimulus vary together.

Since every atmosphere furnishes a different intensity of stimulus, it is fruitless to compare the delays in onset that have been measured in local areas with those found in sweating over the whole body. It is sufficient to point out that all the delays measured are matters of many minutes. It seems doubtful whether any intensity of superficial stimulus of heat can induce the acceleration to a plateau rate of sweating within a time short enough to correspond with a reflex time alone.

Comparable measurements of initiation of full sweating have been made by a rather similar method by W. B. Bean and others at the Armored Medical Research Laboratory. In general outline their results are similar to those here reported.

SUMMARY

1. Rates of evaporative loss were measured in men initially exposed to hot dry air (122°F., 10 per cent humidity). A probable increase in rate of evapora-

tion due to sweating, amounting to $2\frac{1}{2}$ fold, occurred within the 5 minutes before the men could be weighed.

2. The measured acceleration began at 7 minutes in one subject and 12 minutes in another subject. The rates rapidly increased to a maximal rate of 10 fold above that in a cool atmosphere, which was attained in 20 minutes.

3. It is concluded that the promptness of onset of sweating among individuals is no indication of their tolerances or acclimatization to heat.

4. The slow onset of full sweating suggests that the stimulus required is an appreciable increase in the heat content of the body as a whole.

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THE ESTIMATION OF THE CUTANEOUS BLOOD FLOW WITH THE PHOTOELECTRIC PLETHYSMOGRAPH¹

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Similar directional changes in the amplitude of the photoelectrically recorded cutaneous volume pulses (1) and the skin blood flow are readily demonstrated by comparison with other criteria of blood flow such as changes in the skin temperature and with the actual measurements of flow in the digits by the venous occlusion technique. Personal experience indicates that this qualitative correlation holds even under extreme conditions of health and disease. The quantitative correlation between the cutaneous blood flow and the mechanically recorded pulses in the finger has been demonstrated in two independent investigations (2, 3).

The purpose of this paper is to show that this quantitative correlation with the cutaneous blood flow holds also for the photoelectrically recorded cutaneous volume pulses and that one may express this correlation in the form of a simple equation, $F = K \cdot P$, where F is the cutaneous blood flow, P is the amplitude of the photoelectrically recorded cutaneous volume pulse expressed in "filter units" and K is the flow equivalent of the "filter unit".

METHODS. The amplitudes of the photoelectrically recorded cutaneous pulses were calibrated by comparing the pulsatile increase in opacity of the skin resulting from the pulsatile increase in the blood content with the absorption of light produced by inserting a sheet of clear glass (1.1 mm. thick) between the photoelectric cell and the illuminated skin. The recording techniques permitted simultaneous registration of the skin pulses and of the deflection due to the insertion of the "filter" (the term "filter" seemed to be a convenient designation for the sheet of glass). Thus it was possible to express the recorded amplitude of the skin pulse as the ratio of the photoelectric deflection due to the pulse to the photoelectric deflection due to the insertion of the "filter." The method has been described in greater detail elsewhere (1, 4).

The only skin areas available for the calibration of the photoelectrically recorded skin pulses in terms of blood flow are those of the digits. Two methods are available for measuring blood flow in the finger: the adaptation of Stewart's calorimetric method by Mendlowitz (5) and the venous occlusion procedure (2). We have applied each of these methods to the terminal phalanx of the finger. As has been the experience of others, we found that reliable measurements of flow could not be made with the venous occlusion method when the flows were high. Therefore, we have used this method for the measurement only of relatively low rates of flow to which the calorimetric method is probably inapplicable

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for the reason that at low rates of flow an unknown and varying fraction of the thermal loss to the calorimeter is supplied by the thermal reservoir of the phalanx and not by the flow of blood.

In all of the calibration experiments, the photoelectric records of the skin pulses were taken simultaneously with the measurement of flow. In the calorimetric experiments, the flow equivalent of the photoelectric pulse was obtained by dividing the flow by the *average* amplitude (in "filter units") of the skin pulses—the latter were recorded continuously from an adjoining finger during the entire period of the calorimetric determination of blood flow (five to ten minutes). In the venous occlusion experiments, the flow calculated from the slope of the

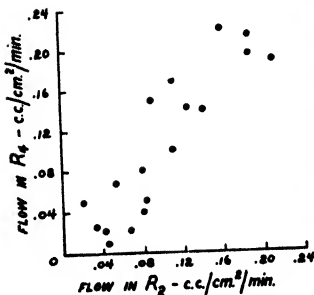


Fig. 1

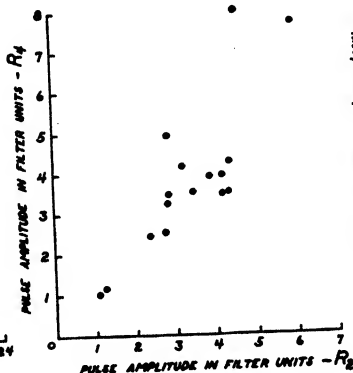


Fig. 2

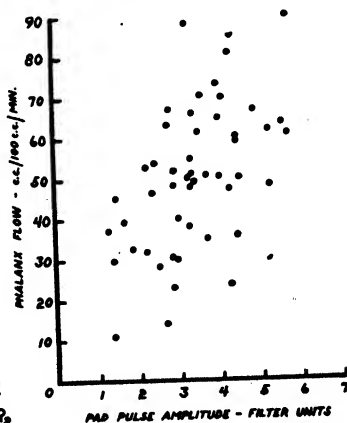


Fig. 2

Fig. 1. Scattergram. Relation of simultaneously measured blood flows in the terminal phalanges of R_2 and R_4 fingers. Calorimetric method used in the measurement of the blood flow.

Fig. 2. Scattergram. Relation of the simultaneously recorded pad pulses in R_2 and R_4 fingers.

Fig. 3. Scattergram. Relation between the pad pulse and the phalanx blood flow (measured by the calorimetric method).

volume curve was compared with the skin pulses (from an adjoining finger) only when the latter remained uniform during the period of the actual determination.

Since the photoelectric records of the skin pulses were obtained from a finger other than that on which the flow measurements were being made, the quantitative similarity in the blood flows in the two fingers was essential to the validity of the calibration experiments. The data of Burton (2) suggested that this quantitative similarity did exist; however, it seemed necessary to establish this point by direct measurements of flow. The data of figure 1 were obtained by simultaneous measurements of blood flow by the calorimetric method on the terminal phalanges of R_2 and R_4 digits. The correlation between the flows in the two fingers is apparent. On examination of the individual data, it became apparent that the correlation between the flows in the two terminal phalanges

was a function of the area of contact of the two phalanges with the water in the calorimeter cups, that is, that phalanx which had a larger area of contact in the calorimeter tended to show the smaller flow per unit area. This source of error in comparing the blood flow in the two fingers may be due to several factors: first, the relation of the magnitude of the thermal reservoir of the phalanx to the area of contact with the water in the calorimeter cup (this has been referred to above); second, topographical inequalities in the cutaneous blood flow which exist over the surface of the phalanx (this point is illustrated below). Unless these items are controlled by immersion of strictly comparable volumes and areas (it has not seemed practicable to do this), greater precision in the correlation of the flows in the two phalanges cannot be expected.

A similar comparison of the pad pulses of R_2 and R_4 fingers recorded simultaneously by photoelectric plethysmographs is exhibited in figure 2. The correlation between the two series of pad pulses is apparent; the deviations in individual sets of measurements are comparable to those shown in the calorimetric measurements of flow. These deviations are probably due to inability to place the individual plethysmographs in exactly comparable positions on the finger pads, thus allowing topographical differences in flow in the pads to express themselves in the photoelectric records.

The correlation between the phalanx blood flow and the pad pulse. The correlation between phalanx blood flow and the pad pulse is shown in figure 3 which is constructed from 52 measurements with the calorimetric method on 21 subjects. It is evident that the scatter of the data is too broad for calibration purposes.

That the correlation between phalanx flow and pad pulse is quite close in any one individual is indicated by the data in table 1 which compares the changes in flow with the changes in pad pulse during a prolonged observation on the same subject. The measurements on flow are for successive periods and the amplitudes of the pad pulses are average values for the corresponding flow period. The changes in flow and also in the pad pulse in a given subject either occurred "spontaneously" or resulted from suitable stimuli which were deliberately presented. In all instances, the pad pulse diminished when the calorimetrically measured flow decreased. Further, there was a definite tendency for the pulse to decrease in direct proportion to the decrease in flow as is indicated by the values of the flow equivalent of the "filter unit." Since the accuracy of the calorimetric measurements of flow is unknown, the small differences in the flow equivalent may have no significance.

The consistency of the correlation, in a given subject, between the phalanx flow and the pad pulse leaves little room for doubt that the cutaneous volume pulse may be employed for the measurement of flow once its flow equivalent has been established. However, the considerable scatter in the values of the flow equivalents in the three subjects of table 1 and also in the data of figure 3 still poses a problem. Since the scatter occurs in the comparison of the data obtained on different subjects, one thinks of such physiological variables as the distensibility of the vessels and topographical differences in the cutaneous blood flow of the finger. It therefore seemed desirable so to modify the calibration experiment

that one might directly compare the amplitude of the pad pulse with blood flow in the pad.

The estimation of the value of K , the flow equivalent of the "filter unit," from the measurements of pad flows and pad pulses. The calorimetric measurements of the blood flow in the finger pad were made in a manner which was essentially similar to the technique used on the terminal phalanx. The much higher rate of flow in the pad as compared with that in the phalanx as a whole is demonstrated in table 2. The phalanx flow includes the pad flow and also that on the dorsal surface. If the latter is much smaller than the pad flow, it will reduce the average phalanx flow. This observation is in agreement with the previously

TABLE 1

Relation between phalanx flow and pad pulse in the same subject

SUBJECT	PHALANX FLOW	PAD PULSE	FLOW EQUIVALENT OF "FILTER UNIT"
	<i>cc./100 cc./min.</i>	<i>filter units</i>	<i>cc./100 cc./min./F.U.</i>
Ca	48.5	5.25	9.25
	36.0	4.42	8.15
	23.2	2.85	7.9
Jo	55.0	3.3	16.7
	48.5	2.94	16.5
	32.7	1.86	17.6
Bo	71.5	6.2	11.5
	50.0	4.5	11.1
	28.2	2.5	11.2

TABLE 2

Comparison of calorimetrically measured blood flows in the finger phalanx and finger pad

SUBJECT	PHALANX FLOW	PAD FLOW
	<i>cc./cm.²/min.</i>	<i>cc./cm.²/min.</i>
R. J.	0.18	0.38
T. J.	0.20	0.25
Jo	0.10	0.32
La	0.07	0.08
	0.12	0.16
	0.18	0.26
Ki	0.10	0.19
	0.11	0.16
	0.15	0.30
	0.16	0.33
Averages.....	0.14	0.24

published photoelectric data which showed that the skin pulses are much larger in the pad than on the dorsum of the finger (1).

The fact of a much higher rate of flow in the pad invalidates the direct calculation of the flow equivalent of the pad skin pulses from the measurement of phalanx flows. Hence, a direct comparison of pad pulses and of pad flows was attempted and is presented in figure 4 which was prepared from 54 observations on 36 subjects. The data show a correlation between the amplitude of the pad pulse and the pad flow but there is considerable scatter in the data. Yet, when the values are recalculated in terms of the flow equivalent of the pulse, approximately 75 per cent of the flow equivalents fall in the range: 0.07-0.11 cc./cm.²/min./filter unit with a mean value of 0.095 cc./cm.²/min./filter unit.

A relation between the magnitude of area of the pad in the calorimeter and the flow equivalent of the pad pulse became apparent on examining the individual

measurements of figure 4. The experiments in which the flows were measured on the smaller pad areas provide larger flow equivalents for the pad pulse. Thus, in table 3, in one series of measurements, the average pad area in the calorimeter was 6.4 cm.² and the average flow equivalent of the filter unit was 0.074 cc./cm.²/min. In another series of measurements, the corresponding values were 2.23 cm.² and 0.10 cc./cm.²/min. Also, one may note in figure 4 that there is a definite tendency for the flow values from the smaller pad areas to place somewhat higher than is true for those from the larger pad areas. Further, one may note that if the higher rates of flow in the small pad areas are extended to the entire pad (the area of which has been estimated as described below), the calculated total

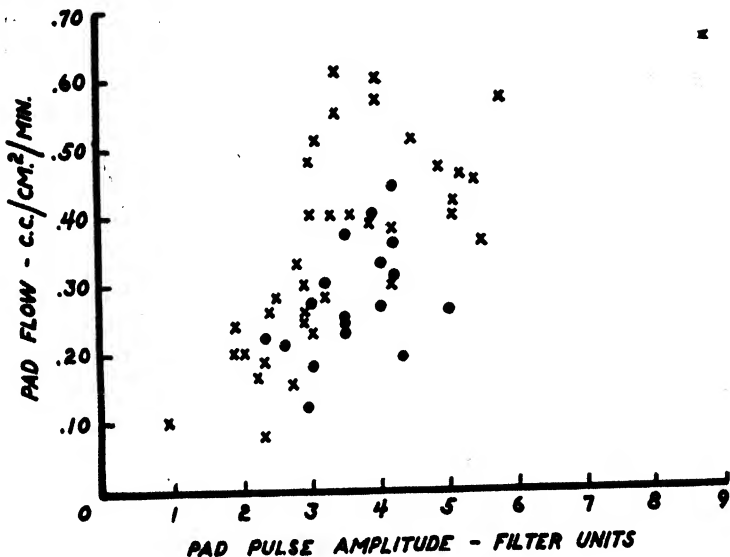


Fig. 4. Scattergram. Relation between pad blood flow (measured by the calorimetric method) and the pad pulse. ● Large pad areas (average: 6.5 cm.²); + small pad areas (average: 2.3 cm.²).

flow in the entire pad actually exceeds the total flow in the phalanx. This is not the case when the average rate of flow in the pad is determined over a much larger area.

These data agree with the anatomical descriptions of the arterial plexus in the pad and also with the volume pulse data (below) in that they indicate topographical differences in the rate of blood flow even in the finger pad. Therefore, unless the pad pulses are recorded precisely from the same area as that in which the blood flow is being measured, the topographical differences in blood flow will introduce considerable error and scatter in the determinations of the flow equivalents of the volume pulse.

The estimation of the value of K, the flow equivalent of the filter unit, from measure-

ments of the average phalanx flow and of the average phalanx skin pulse. Since the measurement of flow in the entire phalanx (by either the calorimetric or venous occlusion methods) yields a value which is the mean of the topographical variations in flow over the various surfaces of the phalanx, the calculation of the flow equivalent of the skin pulse from the phalanx flow requires that one estimate a corresponding average skin pulse from a series of pulse recordings taken from

TABLE 3

The flow equivalent, K, of the filter unit as calculated from pad flow and pad pulse

	PAD AREA†	PAD FLOW	PAD PULSE	K FLOW EQUIVALENT OF F.U.	NUMBER OF OBSERVATIONS
	cm. ²	cc./cm. ² /min.	F.U.	cc./cm. ² /min.	
Averages.....	6.4	0.266	3.63	0.074*	16
Range.....	4.0-9.3	0.12-0.44	2.3-5.0	0.05-0.11	
Averages.....	2.23	0.364	3.61	0.10	40
Range.....	1.6-3.6	0.08-0.65	0.90-8.8	0.035-0.16	

Flow measured calorimetrically.

* "Corrected" value for K = 0.098 (see text).

† Area of pad in calorimeter cup.

various points on the phalanx surface. It is obvious that the average phalanx skin pulse is obtained from the equation

$$(2) \quad P_{PH} = \frac{p_1 \cdot a_1 + p_2 \cdot a_2 + p_3 \cdot a_3 \cdots \text{etc.}}{A_{PH}}$$

where

P_{PH} = average phalanx skin pulse,

A_{PH} = area of phalanx,

$p_1, p_2, p_3 \cdots \text{etc.}$ = amplitudes of the cutaneous volume pulses (expressed in filter units) in the corresponding skin areas, $a_1, a_2, a_3 \cdots \text{etc.}$

In this equation, a_1 or a_2 , or $a_3 \cdots \text{etc.}$ is the extent of the area in which the volume pulses have the same amplitudes.

To determine the average phalanx skin pulse, the skin pulses were recorded from eight different points on the phalanx surface. These points and the corresponding amplitudes of the skin pulses are indicated in table 4 in which the values are averages for 11 subjects. The data illustrate that the surface of the phalanx may be divided into two areas: one which we shall call the pad area, includes the pad and tip of the phalanx, extends well on to the lateral surface of the phalanx and shows relatively large pulses; the other, which we shall call the dorsum area, includes the dorsal aspects of the phalanx and nail and shows relatively small pulses. It is fairly easy to delimit these two areas from each other as the amplitudes of the pulses decrease rapidly as the plethysmograph is moved dorsally along the lateral aspects of the phalanx. The ratio of these two areas varies somewhat in different subjects and therefore requires actual deter-

minations in the individual subjects when precise information is necessary as is the case in these calibration experiments.

It is also obvious from table 4 that the average pulse of the pad area is somewhat less than that recorded at the center of the pad which is the usual position for the application of the plethysmograph; the average difference is such that the average pad pulse is about 75 per cent as large as that recorded at the center of the pad. This fact suggests that a significant correction may be made in the data of table 3 in which the upper series of flow equivalents was calculated by dividing the flow in a relatively large pad area by the pulses which were recorded at the center of the pad. Since the rate of flow in the large pad area is very nearly the average pad flow, it would be more correct to divide it by the average pad pulse. When this correction is introduced into the values of table 3, the value for K becomes 0.098 instead of 0.074. This is in better agreement with

TABLE 4

Topographical differences in blood supply of phalanx as indicated by amplitudes of skin pulses (average values)

POINT OF CONTACT OF PLETHYSMOGRAPH:	PULSE AMPLITUDE IN "FILTER UNITS"
1. Center of pad.....	2.84
2. Palmar surface at crease between 2nd and 3rd phalanges.....	1.27
3. Tip of phalanx.....	1.85
4. Ventro-lateral.....	2.70
5. Lateral.....	2.12
6. Dorso-lateral.....	0.65
7. Dorsal.....	0.55
8. Nail.....	0.60
Pad area (of large pulses).....	7.6 cm. ²
Dorsal area (of small pulses).....	6.4 cm. ²

Number of subjects, 11.

the value for K , 0.10, which was calculated from the data on the smaller pad areas.

Returning to equation (2), it is apparent that the exploration of the phalanx surface indicated in table 4 does not wholly satisfy the requirements of the equation; nor is it practicable to do so. An approximation of the correct value for the average phalanx pulse may be obtained from the following simplification of equation (2)

$$(3) \quad P_{PH} = \frac{A_p \cdot P_p + A_d \cdot P_d}{A_{PH}}$$

where A_p and A_d are the areas of the pad and dorsal surfaces showing large and small pulses respectively. P_p is the average pad pulse as defined above. P_d is similarly the average dorsum pulse. Since it is not practicable to determine directly the values for P_p and P_d while also making flow measurements, it is necessary to substitute for P_p and P_d the values which are in turn calculated

from the recordings of the skin pulses at positions 1 and 7 of table 4; these recordings were taken simultaneously with the flow measurements. The calculation of P_p was done by estimating the value of the ratio, P_p /pulse at 1, from the exploration of the phalanx with the photoelectric plethysmograph either previous to or subsequent to the simultaneous recording of flow and pulses at positions 1 and 7. The value of P_d is usually so nearly equal to the amplitude of the pulse recorded at position 7 (table 4) that the substitution of the latter value for P_d introduces little error. The photoelectric data also permitted calculation of the

TABLE 5

The value of the constant, K, (the flow equivalent of one filter unit) as calculated from the average phalanx flow and the average phalanx pulse (photoelectrically estimated)

SUBJECT	A_p/A_{PH}	A_d/A_{PH}	PAD PULSE AT 1	P_p /PAD PULSE AT 1	P_p	P_d	P_{PH}	F_{PH} *	K*
La.....	0.58	0.42	2.62	0.80	2.09	0.62	1.48	0.15	0.101
Mi.....	0.53	0.47	4.0	1.00	4.00	1.9	3.00	0.28	0.094
Ki.....	0.55	0.45	2.86	0.63	1.80	0.41	1.17	0.13	0.111
T. J.....	0.53	0.47	2.90	1.00	2.90	0.65	1.85	0.20	0.108
Gr.....	0.61	0.39	3.12	0.78	2.44	0.48	1.68	0.156	0.093
Sh.....	0.54	0.46	5.12	0.80	4.10	0.95	2.66	0.180	0.068
Jo.....	0.54	0.46	3.22	1.00	3.22	1.10	2.23	0.177	0.080
A. Br.....	0.49	0.51	4.00	1.00	4.00	0.66	2.30	0.23	0.10
Co.....	0.53	0.47	5.90	0.71	4.20	1.20	2.78	0.23	0.083
Be.....	0.60	0.40	4.60	0.64	2.94	2.20	2.64	0.29	0.110
Average.....									0.093
La.....	0.58	0.42	1.00	0.80	0.80	0.14	0.52	0.048	0.093
Mi.....	0.53	0.47	2.13	1.00	2.13	0.96	1.58	0.163	0.103
Ki.....	0.55	0.45	1.49	0.63	0.94	0.27	0.65	0.069	0.106
T. J.....	0.53	0.47	1.31	1.00	1.31	0.32	0.84	0.052	0.062
Average.....									0.091

* Values in cc./cm.² min.

P_p = average pad pulse; P_d = average dorsum pulse; A_p = pad area;

A_d = dorsum area; A_{PH} = area of phalanx; P_{PH} = average phalanx pulse;

F_{PH} = average phalanx flow.

values of the ratios, A_p/A_{PH} and A_d/A_{PH} . It is assumed that the values for these various ratios hold during the periods of recording of the blood flows and of the skin pulses.

The average phalanx flow (F_{PH}) was estimated by the calorimetric method in the first ten subjects of table 5 in whom the finger vessels were dilated at the time of measurement of flow. The venous occlusion method was used in the next four subjects of table 5 who had also been used in the calorimetric measurements; the finger vessels of these four subjects were constricted at the time of the measurements. Simultaneously with the measurements of blood flow, recordings were taken from another finger of the skin pulses on the pad at position

1 and on the dorsum at position 7 (of table 4) and the values of P_r and of P_d calculated as above. The value for the average phalanx pulse was then calculated according to equation (3) and the value of K , the flow equivalent of the filter unit, is obtained from the quotient, average phalanx flow divided by average phalanx pulse. The average value of K was 0.093 cc./cm.²/min. for the calorimetric measurements of flow and 0.091 cc./cm.²/min. for the flow measurements made by the venous occlusion method. These values for K are in agreement with those calculated from pad flows and pad pulses (table 3). The scatter of the individual values for K was fairly narrow; 85 per cent of the values were within 20 per cent of the average value.

DISCUSSION. The correlation in any one subject between the rate of blood flow in the terminal phalanx of the finger and the amplitudes of the photoelectrically recorded cutaneous volume pulses in the finger pad indicates that if the blood flow and the skin pulses are measured in the same skin area, a quan-

TABLE 6

Summary: Average values of K , the flow equivalent of the filter unit, as derived from the various calibration experiments

METHOD	K
	<i>cc./cm.²/min./filter unit</i>
Pad flow and pad pulse:	
Large pad area.....	0.098
Small pad area.....	0.100
Average phalanx flow and average phalanx pulse:	
Calorimetric data.....	0.093
Venous occlusion data.....	0.091
Average.....	0.095

titative correlation will be found which is expressed in the equation, $F = K \cdot P$. This possibility has been tested experimentally in this paper in two types of calibration experiments: in the first series, the blood flow and the skin pulses were measured in the finger pad; in the second series, the average phalanx pulse was calculated from an exploration of the phalanx surface and correlated with the average phalanx flow. The results are summarized in table 6 from which we may infer that the value of K is approximately equal to 0.10 cc./cm.²/min. The values for K derived from measurements on the entire phalanx are slightly less than this, but the average of all values is so close to this figure that its acceptance as the measured value of K seems warranted.

This value for K applies to the finger in normal subjects within a wide range of blood flows; we could discover no effect of high or of low flows on the value of K . This observation agrees with the linear relation of the finger's volume pulse and blood flow as demonstrated in the data of Burton (2) and of Goetz (3). However, the lowest value for the phalanx blood flow, (0.048 cc./cm.²/min.—table 5), in the range of values on which these statements rest is still some ten times as

great as the average ($0.005 \text{ cc./cm.}^2/\text{min.}$), for the basal skin flow of the body (6). It is not possible to reduce the phalanx flow to this level by even very strong vasoconstrictor reflexes; their reinforcement by the direct action of cold introduces such changes in the dynamics of the finger's circulation as to cast serious doubt on a calibration procedure. We therefore hesitate to apply quantitatively the flow equivalent of the "filter unit" to other skin areas where the level of blood flow is very low.

Possibly, the flow equivalent of the "filter unit" shows some variation from subject to subject but our data are inconclusive on this point. Repetition of experiments on some subjects resulted in as large variations in the value for K as are exhibited in different subjects. These variations may represent the operation of the various experimental errors in the calibration experiments, e.g., errors in the measurement of blood flow and errors due to topographical inequalities in blood flow rather than any real changes in the value of K .

There was no apparent effect of the pulse rate (range: 57–100 in these experiments) on the flow equivalent of the skin pulse. Neither did the form of the pulse wave exercise any recognizable influence. However, the effects of extreme variations in either factor have not been quantitated.

SUMMARY

The problem of quantitating the records of the cutaneous volume pulses as inscribed by the photoelectric plethysmograph, in terms of cutaneous blood flow, is examined:

1. The technique of quantitative recording of the cutaneous volume pulses by the photoelectric plethysmograph is described. The amplitudes of these pulses so recorded are expressed in terms of an arbitrary unit which is called the "filter unit." This unit is defined.

2. The quantitative correlation between variations in the blood flow in the terminal phalanx of the finger and in the amplitude of the photoelectrically recorded cutaneous volume pulses of the finger pad is demonstrated by direct measurements (table 1).

3. The flow equivalent of the "filter unit" which is used to express the amplitudes of the photoelectrically recorded cutaneous volume pulses was estimated from two types of calibration experiments: (1) The simultaneous measurement of blood flow and of pulses in the finger pad (fig. 4 and table 3). The higher rate of blood flow in the finger pad than in the phalanx as a whole (table 2) demonstrated that topographical inequalities in rates of blood flow exist over the surface of the phalanx (table 4). This fact invalidated the direct calibration of pad pulses by measurements of phalanx flow. (2) The simultaneous measurement of the average blood flow in the terminal phalanx of the finger and the estimation of the average cutaneous volume pulse of the phalanx by the exploration of the surface of the phalanx with the photoelectric plethysmograph (table 5). The fact of topographical inequalities in the blood flow in the various areas of the phalanx is further demonstrated by these data.

The flow equivalent of the "filter unit" thus determined is approximately

equal to 0.10 cc./cm.² of skin/minute (table 6). This value may be applied to the photoelectric records of the cutaneous volume pulses in order to convert them into reasonably quantitative measurements of cutaneous blood flow.

The approximate equality in the rates of blood flow in neighboring fingers of normal subjects may be inferred from the photoelectric data (fig. 2) and the flow measurements (fig. 1).

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